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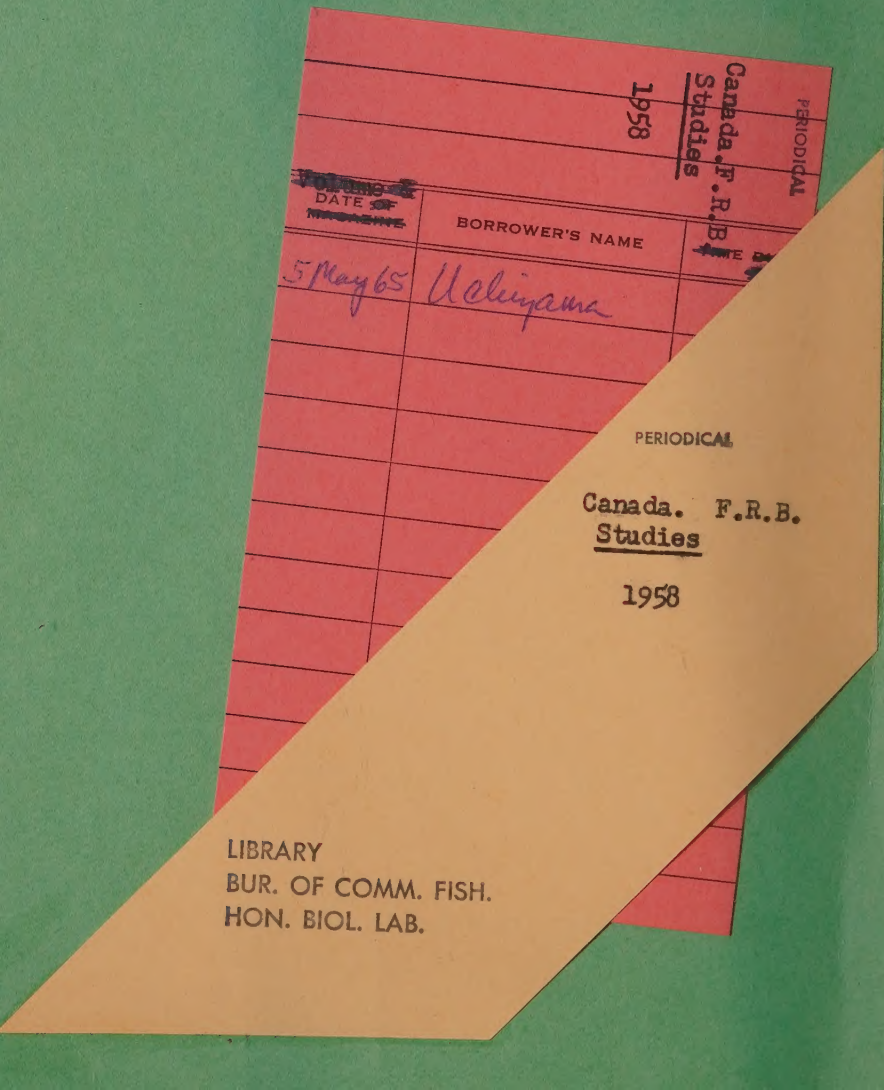
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1958

FROM THE STATIONS OF THE FISHERIES RESEARCH BOARD OF CANADA

—CONTENTS—

512	Variation in number of dorsal spines in the brook stickleback, *Eucalia inconstans*	G. H. Lawler
513	Boring sponges, *Cliona* species, of eastern Canada, with a note on the validity of *C. lobata*	Frederick E. Warburton
514	Comparative effectiveness of tetracycline antibiotics for fish preservation	B. A. Southcott E. G. Baker J. W. Boyd H. L. A. Tarr
515	Reproduction of fused larvae in the boring sponge, *Cliona celata* Grant	Frederick E. Warburton
516	Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth	Robert A. MacLeod E. Onofrey
517	Biochemistry of fishes	H. L. A. Tarr
518	Epidemic shellfish poisoning in New Brunswick, 1957	R. M. Bond J. C. Medcof
519	Dolphins in Newfoundland waters	David E. Sergeant
520	Biochemical studies on sockeye salmon during spawning migration. I. Physical measurements, plasma cholesterol, and electrolyte levels	D. R. Idler H. Tsuyuki
521	Nutrition and metabolism of marine bacteria. VII. Growth response of a marine flavobacterium to surface active agents and nucleotides	Robert A. MacLeod H. Hogenkamp E. Onofrey
522	The acid-soluble nucleotides of salmon liver	H. Tsuyuki Violet M. Chang D. R. Idler
523	Sodium phosphate crystals on salt fish	W. J. Dyer Doris I. Fraser J. R. Dingle
524	Preservation of fresh fish	H. L. A. Tarr
525	Effects of DDT spraying in New Brunswick on future runs of adult salmon	C. J. Kerswill
526	Lingcod muscle purine nucleoside phosphorylase	H. L. A. Tarr
527	Some sociological effects of quota control of fisheries	J. L. Hart
528	Regulation of the lobster fishery	D. G. Wilder
529	Regulation of the Atlantic salmon fisheries	C. J. Kerswill
530	Some notes on a collection of polychaeta from the northeast Pacific south of latitude 32° N	E. Berkeley C. Berkeley
531	Amino acid analysis by horizontal paper chromatography	Jacqueline Chartier F. W. van Klaveren G. Vaillancourt

532 Should we pre-wrap fresh fillets in consumer packages? - - - - - - - C. H. Castell

533 Observations on the function of sodium in the metabolism of a marine bacterium - - - - - - - - - - - - - - - - - - Robert A. MacLeod, C. A. Claridge, Aiko Hori, J. F. Murray

534 Ribonuclease activity of fish muscle extracts - - - - - - - - - - Neil Tomlinson

535 Some principles involved in regulation of fisheries by quota - - - - - W. E. Ricker

536 Biochemical studies on sockeye salmon during spawning migration. II. Cholesterol, fat, protein, and water in the flesh of standard fish - - D. R. Idler, I. Bitners

537 Biochemical studies on sockeye salmon during spawning migration. III. Changes in the protein and non-protein nitrogen fractions in muscles of migrating sockeye salmon - - - - - - - - - - - - - - D. W. Duncan, H. L. A. Tarr

538 The manner in which the sponge *Cliona* bores in calcareous objects - - - Frederick E. Warburton

539 Biochemical studies on sockeye salmon during spawning migration. IV. The non-protein nitrogenous constituents of the muscle - - - - - - J. D. Wood

540 The keeping time of wrapped fillets treated with an antibiotic - - - - C. H. Castell

541 A direct-current electrofishing apparatus using separate excitation - - - A. R. Murray

542 Nitrogen excretion in some marine teleosts - - - - - - - - - - J. D. Wood

543 The identity of the species of *Lepeophtheirus* (Copepoda) parasitic on Pacific salmon (genus *Oncorhynchus*) and Atlantic salmon (*Salmo salar*) - - L. Margolis

544 A new species of *Lecithophyllum* from north Pacific fishes with a consideration of the taxonomy of the genera *Lecithophyllum*, *Aponurus*, and *Brachadena* (*Trematoda: Hemiuridae*) - - - - - - - - - - - - - - - - L. Margolis

545 Studies on *Mytilus edulis* L. of the "Calanus" expeditions to Hudson Bay and Ungava Bay. "Calanus" Series No. 16 - - - - - - - - - - I. Lubinsky

546 Marine sterols. V. Isolation of 7,24(28)-ergostadien-3β-ol from starfish - U. H. M. Fagerlund, D. R. Idler

547 Variations in the sodium and potassium content of the muscle tissue of Pacific salmon with particular reference to migration - - - - - - - - Robert A. MacLeod, R. E. E. Jonas, J. R. McBride

548 Observations on marine birds off southwestern Nova Scotia - - - - - David M. Scott

549 The genus *Prorocentrum* Ehrenberg. Morphodynamics, protoplasmatic structures, and taxonomy - - - - - - - - - - - - - - - - Adam Bursa

550 The freshwater dinoflagellate *Woloszynskia limnetica* n. sp. membrane and protoplasmic structures - - - - - - - - - - - - - - - - Adam Bursa

551 The origin and speciation of *Oncorhynchus* - - - - - - - - - - Ferris Neave

551A The use of antibiotics for the control of spoilage in the east coast fisheries - C. H. Castell, Maxine F. Greenough

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VARIATION IN NUMBER OF DORSAL SPINES IN THE BROOK STICKLEBACK, EUCALIA INCONSTANS[1]

G. H. LAWLER

Abstract

The brook stickleback, *Eucalia inconstans* (Kirtland), is usually described as possessing five or six dorsal spines and the species is commonly called the five-spine stickleback. In Heming Lake, Manitoba, fish with six dorsal spines predominate. The occurrence of sticklebacks with a high number of dorsal spines is noted from other Manitoba lakes and to a lesser extent in samples from some Ontario lakes. Indications are that the dorsal spines are more numerous in fish from the Hudson Bay drainage than from the Great Lakes region. An increase in the number of dorsal spines with increasing latitude is apparent.

Introduction

During the course of an investigation of the fishes of Heming Lake, Manitoba, it was observed that the brook stickleback, *Eucalia inconstans* (Kirtland), had an unusually high average number of dorsal spines. Hubbs and Lagler (2) described *Eucalia inconstans* as possessing five or six spines while Dymond (1) stated that usually five spines are present. No records of spine counts from different geographical areas were found and consequently specimens from several localities in Manitoba and Ontario were examined to determine whether the variation observed in the Heming Lake sticklebacks was also characteristic of these other areas.

Materials and Discussion

Dorsal spine counts were made on 140 specimens of *Eucalia inconstans* from Heming Lake, ranging in size from 39 to 76 mm. total length. Of those examined, 66% had six dorsal spines, 31% had five spines, and 3% had seven spines (Table I). No sticklebacks were found that had only four dorsal spines.

Counts made on 202 specimens of *Eucalia inconstans* representative of various parts of Manitoba are shown in Table I. The ratio of dorsal spines was four-spine—7; five-spine—156; six-spine—39. The number of six-spined fish was considerably higher than those with four spines and in one sample they constituted 54% of the sample. In other samples those sticklebacks with six spines made up 37, 36, and 25% of the samples, respectively. Of 12 samples of Manitoba sticklebacks examined only two did not contain fish that possessed six dorsal spines.

It was considered that possibly the higher numbers of dorsal spines in *Eucalia inconstans* were more prevalent in Manitoba so the scope of the survey was broadened to include the Province of Ontario. Samples from several Ontario localities ranging from the St. Lawrence River region to

[1] Manuscript received November 20, 1957.
Contribution from Biological Station, London, Ontario.

TABLE I

VARIATION IN NUMBER OF DORSAL SPINES IN *Eucalia inconstans* FROM SEVERAL REGIONS OF MANITOBA AND ONTARIO, CANADA, ARRANGED IN ORDER FROM SOUTH TO NORTH

Locality	Lat.	Long.	IV	V	VI	VII	Percentage with six spines	No. on map
Manitoba								
Long River	49° 8′	99° 00′	0	7	4		36	1
Pembina River	49° 13′	99° 42′	0	3	0		0	17
Mary Jane Creek	49° 13′	98° 43′	0	10	2		16	0
Hanson's Creek	49° 45′	95° 15′	0	6	2		25	2
Rennie Creek	49° 45′	95° 35′	0	24	2		8	5
LaSalle River	49° 54′	97° 46′	0	17	10		37	6
Cook's Creek	50° 00′	98° 5′	0	9	1		10	3
Marsh Lake	50° 15′	97° 15′	1	4	1		16	4
Clear Lake	50° 41′	100° 00′	4	54	11		16	7
Branch of Vermillion River	51° 10′	100° 3′	2	17	0		0	8
South Pine River	51° 47′	100° 20′	0	5	6		54	9
Heming Lake	54° 53′	101° 7′		43	92	5	66	10
Ontario								
Pickering Twp. near Audley	43° 55′	79° 00′	1	50	6		11	11
Housic Creek	45° 00′	75° 15′	1	12	1		7	12
Cache Lake	45° 32′	78° 35′	5	38	2		4	13
Cedar Lake	49° 20′	88° 10′	2	14	3		16	14
Fairloch Lake	49° 30′	88° 00′	4	19	0		0	15
Bog Lake near Fort Albany	52°	81°	1	17	12		40	16

James Bay were examined and spine counts recorded (Table I). The percentage of sticklebacks with six spines ranged from 7 to 40 and only one sample did not contain fish with six dorsal spines. Only the northern sample from the James Bay region had an unusually high proportion of six-spined fish. The percentage of *Eucalia inconstans* from Ontario waters with six spines was slightly lower than in Manitoba.

The percentage of the samples containing six-spined sticklebacks from Manitoba and Ontario were plotted on a map (Fig. 1) to see if a distributional pattern was evident. The limited distributional records indicate that the dorsal spines of *Eucalia inconstans* are more numerous in fish found in the Hudson Bay drainage than in the Great Lakes region. Also it would appear that there is an increase in the number of dorsal spines with increasing latitude as the northernmost fish had the greatest number of dorsal spines. The latter statement tends to agree with the findings of Walters (4) whose data on the nine-spine stickleback, *Pungitius pungitius*, revealed a tendency, in ranging from colder to warmer climates, for lower spine counts to appear, and higher spine counts to disappear, accompanied by a downward shift in the mode and mean. He states "it appears as though some factor in the environment

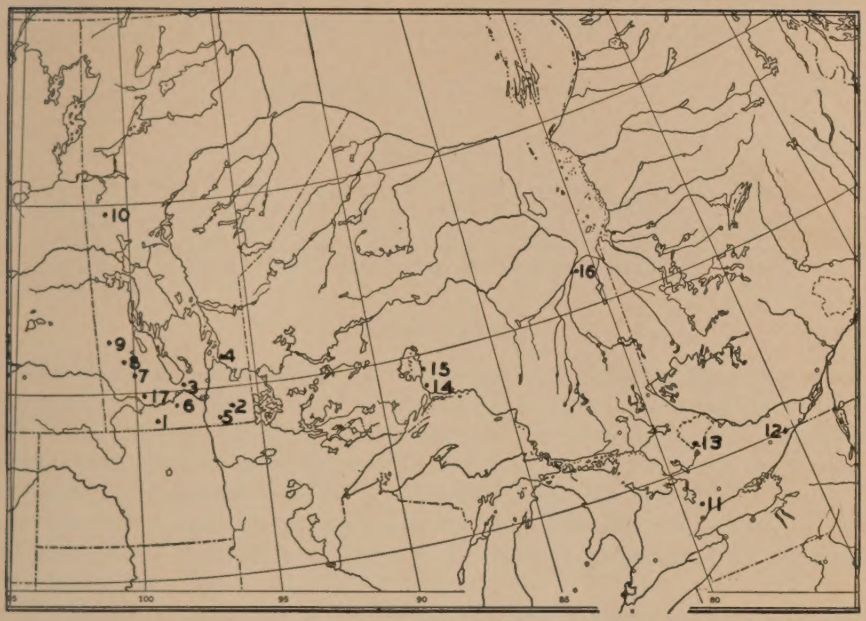

FIG. 1. Outline map of Manitoba and Ontario showing localities of collections of *Eucalia inconstans*.

were selectively eliminating those individuals with spine counts not adaptive to the locality", which possibly explains why no sticklebacks with four spines were collected at Heming Lake. Further evidence of latitudinal variation in spine counts is shown by Vladykov (3).

Acknowledgments

I am indebted to Dr. W. B. Scott, Royal Ontario Museum, Division of Zoology and Paleontology, who loaned specimens of Ontario sticklebacks for examination. Mr. J. J. Keleher of this station made available specimens collected by him in Manitoba and offered advice in the preparation of this manuscript.

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BORING SPONGES, CLIONA SPECIES, OF EASTERN CANADA, WITH A NOTE ON THE VALIDITY OF C. LOBATA[1]

FREDERICK E. WARBURTON[2]

Abstract

Cliona celata occurs in Prince Edward Island; *C. lobata* in Prince Edward Island and on the Gulf shore of New Brunswick; *C. vastifica* in the Bay of Fundy, off Sable Island, off Prince Edward Island, and off Newfoundland. Intraspecies grafts of *C. celata* and *C. lobata* succeeded, but interspecies grafts failed. This should remove doubt about the taxonomic distinctness of these two species.

Introduction

The common boring sponge (*Cliona celata*) was reported from the north shore of Prince Edward Island by Lambe (3) in 1896. Since then little that is new has been published on the occurrence of the genus in eastern Canadian waters. In discussions of the oyster fishery, a few writers have mentioned *C. celata* in Malpeque Bay, P.E.I. (Stafford, 1913 (8); Needler, 1941 (6)), and the only Canadian material used by Old (7) in his 1941 review of the Atlantic North American species was *C. celata* collected by Needler from Malpeque Bay.

During an investigation of the biology of boring sponges carried on by the Fisheries Research Board of Canada from its Biological Sub-Station, Ellerslie, P.E.I., two other species were found in various localities. Collections were made by A. A. Skinner, L. M. Dickie, J. C. Medcof, and the writer. Identification of species was based on spiculation, using the key prepared by Old (1941 (7)).

Distribution

Cliona celata Grant (1826 (1))

This is the commonest species in Malpeque Bay, and occurs frequently on other parts of the north shore of Prince Edward Island, often well up in estuaries. It has been found in the shells of living and dead oysters (*Crassostrea virginica*), mussels (*Mytilus edulis*), slipper limpets (*Crepidula fornicata*), and barnacles (*Balanus* sp.), and in empty shells of bar clams (*Spisula solidissima*), quahaugs (*Venus mercenaria*), and soft-shelled clams (*Mya arenaria*), as well as in limestone pebbles. Probably it will inhabit any type of shell or other calcareous substance available in its environment.

Cliona vastifica Hancock (1849 (2))

This species is common in scallops (*Placopecten magellanicus*) off Digby, N.S., at St. Andrews, N.B., and off Sable Island. It also occurs in empty

[1]Manuscript received November 18, 1957.
Contribution from the Fisheries Research Board of Canada, Biological Station, St. Andrews, N.B.
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conch shells (*Neptunea* sp.) at Digby, and in conchs and periwinkles (*Littorina littorea*), washed ashore on the north shore of Prince Edward Island. One specimen, in an Iceland scallop shell (*Chlamys islandicus*), was obtained from Hare Bay, Nfld.

Cliona lobata Hancock (1849 (2))

This sponge was found in the shells of oysters from the Miramichi area and from Shippegan, N.B., and in oysters from Malpeque Bay, P.E.I. However, in Prince Edward Island, it is less common than *C. celata*. A specimen from Malpeque Bay had bored in a *Polinices* shell occupied by a hermit crab.

Taxonomic Validity of *C. lobata*

De Laubenfels (1949 (5)) mentions that many authors, following Topsent (1900 (9)), consider *C. lobata* synonymous with *C. celata*, since it has been reported that young specimens of *celata* contain spirasters, which are one of the diagnostic features of *lobata* given by Hancock (1849 (2)), and used as a key character by Old (1941 (7)), who maintains the distinction between the two species. The writer has never found spirasters in *celata* at any size, but found them consistently numerous in all specimens of *lobata*.

Wilson (1911 (10)) found that cells of *Microciona prolifera*, dissociated by being pressed through bolting silk, would reconstitute themselves into miniature sponges, but that cells of *Lissodendoryx* added to the suspension formed separate reconstitution bodies. De Laubenfels (1927 (4)) tried all possible bispecific mixtures of cells of three species of *Pachychalina* forced through bolting silk; none of the mixtures formed functional sponges, although dissociated cells of individual species metamorphosed successfully.

This physiological approach would seem to be a useful accessory to the usual methods of sponge taxonomy. The writer has adapted and simplified it to test the debated distinction between *C. celata* and *C. lobata*. Fragments of *C. celata* were taken from different shells and piled together in a dish of sea water. These became grafted together, and within a few days the fused mass remodelled itself into a single sponge, with a functional canal system. Fragments of *C. lobata* behaved in the same way. However, it proved impossible to graft fragments of *C. lobata* to fragments of *C. celata*. When fragments of the two types were piled together, whichever formed the majority of the mass remodelled itself into a functional sponge, but the fragments of the other species remained separate as rounded, structureless masses. The writer believes that these experiments are strong evidence for the validity of the specific distinction between *C. celata* and *C. lobata*.

Acknowledgments

The writer would thank all his associates at the Ellerslie Sub-Station for their assistance with this work; and Dr. J C. Medcof for criticizing the manuscript of this paper.

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No. 2, Pages 108–110]

Comparative Effectiveness of Tetracycline Antibiotics for Fish Preservation[a]

(Manuscript received May 15, 1957)

**B. A. Southcott, E. G. Baker,
J. W. Boyd, and H. L. A. Tarr**

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Technological Station, Vancouver, B.C.

Independent investigations conducted in two widely-separated laboratories six or seven years ago *(5, 11, 12)* showed that the most satisfactory antibiotics for flesh food preservation were, in decreasing order of effectiveness, chlortetracycline (CTC), oxytetracycline (OTC) and chloromycetin. In view of these results, subsequent research in this field was largely carried out with CTC. However, the recent approval for use of CTC for poultry preservation in the United States and Canada, and for fish preservation in Canada, has undoubtedly stimulated interest in further possible applications of antibiotics for food use. This in turn has led to a need for further detailed research on the comparative effectiveness of tetracycline antibiotics for flesh food preservation. In general, the more recent comparative studies with poultry *(1, 6)* and with fish *(3, 13)* have shown that CTC is a more effective preservative than OTC or tetracycline (TC), though recent studies in which salmon were stored in cooled sea water indicated that

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the three tetracycline antibiotics possessed similar activity *(7, 8)*. Since our own early comparative data with CTC and OTC were somewhat limited *(12)*, and since we had only conducted a few tests with TC, a comparative study of the effectiveness of the three tetracycline antibiotics was made.[b]

EXPERIMENTAL PROCEDURES

The antibiotics were compared under 3 different conditions of application as follows. Lingcod *(Ophiodon elongatus)* were used in most experiments as these fish spoil more rapidly than certain other commercially used North Pacific fish.

Treatment of fillets. Fish were filleted under clean conditions, the fillets skinned and pieces of fairly uniform size were then prepared. The latter were dipped 1 minute in water with and without 5 p.p.m. of tetracycline antibiotic, drained for 2 minutes on clean, wire racks, and stored at 0°, 4° or 10° C. in polyethylene bags. A single fillet was examined at each sampling interval.

Storage of eviscerated fish in ice. Ice containing CTC, OTC and TC was prepared in 300-lb. blocks in a commercial ice plant using carboxymethylcellulose as distributing agent *(2, 4)*. Each block received a mixture consisting of carboxymethylcellulose (Hercules "High Viscosity 70" <100 mesh), C.P. sodium chloride and clinical grades of the antibiotics as hydrochlorides in amounts sufficient to give final concentrations of 100, 300 and 5.0 p.p.m., respectively. Strictly fresh fish, usually in *rigor mortis*, were used in these and subsequent experiments. They were lightly rinsed in fresh water, and were then iced in insulated clean galvanized boxes. At intervals a single fish was removed, filleted on clean paper, and the fillets stored 18 hr. at 10° C. in closed polyethylene bags in order to accelerate bacterial spoilage.

Storage of eviscerated fish in refrigerated brine. Sea water which is maintained at about −1° C. has been found to be an excellent means of maintaining the quality of fresh fish during transport and storage *(9)*. In the present work, since full strength sea water was not readily available, 3% solutions of sodium chloride with and without 1.5 p.p.m. of the tetracycline antibiotics were used. Twenty-litre portions were added to large, covered plastic containers, eviscerated fish immersed therein, and the temperature maintained between −1° and 0° C. In one experiment a stream of air was slowly bubbled through the solutions in an attempt to counteract the anaerobic conditions which prevail under these storage conditions. Sampling and incubation techniques were the same as those used with the iced fish.

Bacterial counts. Direct counts were made by the procedure used routinely in this laboratory. For viable counts dilutions

[b] Since this paper was presented the more recent literature on use of antibiotics in fish preservation has been reviewed *(10)*.

of the blended flesh were made using 3% sodium chloride solution. Bacto nutrient agar containing 3% sodium chloride was used for preparation of the plates and an incubation period of 2 days at 25° C. was employed.

RESULTS AND DISCUSSION

The experiments were designed purposely to maintain the antibiotic concentrations used at fairly low levels such as would be consistent with general commercial practice. Results obtained in the fillet dipping experiments (Tables 1, 2 and 3) show that CTC was considerably and consistently more effective than either OTC or TC, irrespective of the storage temperatures employed. Ice containing CTC proved more effective than ices containing OTC or TC as evidenced by both lower direct and viable bacterial counts and by odour of the fish (Table 4).

Results in the case of the chilled salt solution storage experiments were complicated and somewhat confusing. The first experiment, in which rock cod (*Sebastodes* sp.) were stored in non-aerated solutions (Table 5), brings out several important facts. There was, in the first place, no consistent agreement be-

TABLE 1

Effect of various antibiotic treatments on the bacterial content of lingcod fillets stored at 0° C. (32° F.)

Days stored	Bacteria per g. (x10⁶) by direct count after immersing fillets in the following solutions			
	H_2O	5 p.p.m. CTC	5 p.p.m. OTC	5 p.p.m TC
5	<0.5	<0.5	<0.5	<0.5
8	13	<0.5	5	7
12	190	43	230	180
15	230	70	270	380

TABLE 2

Effect of various antibiotic treatments on the bacterial content of lingcod fillets stored at 3° C. (37.5° F.)

Days stored	Bacteria per g. (x10⁶) by direct count after immersing fillets in the following solutions			
	H_2O	5 p.p.m. CTC	5 p.p.m. OTC	5 p.p.m TC
7	130	4.8	77	66
8	330	31	160	94
10	480	65	450	380
13	430	230	370	430

TABLE 3

Effect of various antibiotic treatments on the bacterial content of lingcod fillets stored at 10° C. (50° F.)

Days stored	Bacteria per g. (x 10^6) by direct count after immersing fillets in the following solutions			
	H_2O	5 p.p.m. CTC	5 p.p.m. OTC	5 p.p.m TC
4	110	14	65	54
5	650	81	620	480
6		100	700	690

tween the total and viable bacterial counts, though the direct counts were always higher. All 3 tetracycline antibiotics gave protection against development of unpleasant off odors, the improvement here being more marked at the 21-day storage interval with CTC than with OTC or TC. With CTC the direct bacterial counts were somewhat lower than those of

TABLE 4

Effect of storage of eviscerated lingcod in ordinary ice and ice containing antibiotics on their total and viable bacterial content and odour

Days stored	Bacteria per g. (x 10^6) and odour ratings[1]							
	No additive		5 p.p.m. CTC		5 p.p.m. OTC		5 p.p.m. TC	
	Direct count	Viable count	Direct count	Viable count	Direct count	Viable count	Direct count	Viable count
14	270	47	6.9	1.4	81	22	91	
	stale		fresh		fresh		fresh	
17	390	30	30	3.8	230	17	220	27
	stale		very slightly stale		slightly stale		slightly stale	
20	420	46	86	10	210	91	250	120
	extremely putrid		slightly stale		putrid		putrid	

[1] The following arbitrary odour rating in order of preference has been employed in this and subsequent tables: fresh<very slightly stale <slightly stale<stale<putrid<extremely putrid.

the control fish, but no important differences in viable counts were detected. With both OTC and TC the viable counts were consistently higher than those of the controls.

Another similar experiment (Table 6) was made with lingcod in order to ascertain the possible influence of aeration on the effectiveness of CTC. The results showed a definite organoleptic and bacteriological improvement which, as far as the former was concerned, continued until the fish had been stored

TABLE 5

Effect of storage of eviscerated rock cod at −1° to 0° C. in 3% NaCl solutions, with and without added antibiotics, on their total and viable bacterial content and odour

Days stored	Bacteria per g. (x10⁶) and odour rating							
	No additive		1.5 p.p.m. CTC		1.5 p.p.m. OTC		1.5 p.p.m. TC	
	Direct count	Viable count	Direct count	Viable count	Direct count	Viable count	Direct count	Viable count
21	49 extremely putrid	0.80	21 fresh	0.15	49 slightly stale	8.8	54 slightly stale	17
25	73 extremely putrid	0.74	24 slightly stale	0.89	53 slightly stale	5.6	52 slightly stale	13
28	84 extremely putrid	0.71	23 stale	0.90	62 stale	16	58 stale	18

20 days. Total bacterial populations were, in general, lower with fish stored in the CTC brine, but the spectacular differences encountered in the fillet dipping tests were not demonstrable. Aeration did not exert any important effect, but this technique may not have been sufficient to change the rather anaerobic conditions which appear to exist in the fish themselves.

Overall results of these trials definitely demonstrate the superiority of CTC over OTC and TC when these are applied in fillet dipping or fish icing procedures. Results with 3% salt solution storage also tend to

TABLE 6

Effect of storage of eviscerated rock cod at −1 to 0° C. in aerated and non-aerated 3% NaCl solutions, with and without added CTC, on their total bacterial content and odour

Days stored	Bacteria per g. (x10⁶) and odour rating			
	No additive, non-aerated	No additive, aerated	1.5 p.p.m. CTC non-aerated	1.5 p.p.m. CTC aerated
13	42 extremely putrid	150 putrid	4.5 fresh	3.1 fresh
15	29 extremely putrid	46 putrid	28 fresh	26 fresh
16	20 extremely putrid	88 putrid	21 fresh	15 fresh
17	46 extremely putrid	55 putrid	5.3 fresh	44 fresh
20	95 extremely putrid	80 extremely putrid	98 fresh	130 fresh

demonstrate the somewhat greater effectiveness of CTC. However, under this latter condition the whole spoilage picture appears to be complicated. Thus, the high total bacterial counts as compared with the low viable counts indicate that bacteria may be rapidly dying off. Spoilage in chilled salt solutions is characterized by development of unpleasant and characteristic odors which suggest presence of diethyl sulfide and hydrogen sulfide. Further experiments have been initiated in order to examine more thoroughly the changes which occur in fish held in this comparatively anaerobic environment, and in these anaerobic viable bacterial counts will also be made.

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Reproduction of Fused Larvæ in the Boring Sponge, *Cliona celata* Grant

Larvæ of the sponge *Lissodendoryx carolinensis* fuse with each other if brought together in culture dishes, and some of the compound larvæ metamorphose[1]. Nevertheless, one would rarely expect such an event in Nature, as most sponge larvæ are released a few at a time, and disperse rapidly. In *Cliona celata*, however, I have observed successful reproduction by fused larvæ in a much more natural environment than a culture dish.

Throughout the summer of 1955, I kept several oyster shells containing boring sponges in an asphalt-lined wooden aquarium through which seawater constantly flowed. The water was pumped from the bottom of the Bideford River, Prince Edward Island, an estuary in which *Cliona* was growing, and did not change appreciably in temperature or lose suspended matter during its passage to the aquarium. *Microciona prolifera*, a sponge much more sensitive to stale water than *Cliona*, lived indefinitely and reproduced freely in a similar tank.

Under these conditions, *C. celata* (which is oviparous) may discharge tens of thousands of eggs from a large osculum in a few hours. The eggs are sticky, and adhere to each other and to the substratum in an irregular flattened mass. Diatoms, the shells of larval molluscs, silt and other debris rapidly accumulate on and in the mass, so that on hatching many of the larvæ are temporarily trapped, able to wander among the interstices of the egg mass but delayed in their escape to open water. In their wanderings, the larvæ sometimes come together in twos or threes and fuse. Fusion is without apparent regard for polarity, so the swimming movements of compound larvæ are often merely ineffective gyrations and many perish within the tangle of debris. A few escape, however, since two such compound larvæ metamorphosed successfully into single sponges, with single oscula, and began boring in crystals of calcite placed near an egg mass.

It seems quite possible, then, that *Cliona* larvæ occasionally metamorphose after fusion, in their normal environment. The natural occurrence of larval fusion, previously dismissed as a purely experimental phenomenon, emphasizes its interest to students of individuation and related problems of theoretical morphology.

This observation is part of a study of *Cliona* conducted at the Fisheries Research Board of Canada's Biological Sub-Station at Ellerslie, Prince Edward Island, which will be more completely reported elsewhere. I thank other members of the Sub-Station staff for their help.

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NUTRITION AND METABOLISM OF MARINE BACTERIA. III. THE RELATION OF SODIUM AND POTASSIUM TO GROWTH

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INTRODUCTION

A number of marine bacteria have been found to require both Na^+ and K^+ for growth (MacLeod et al., '54, '56). Although many bacteria have been shown to require K^+ (Haynes et al., '54) the need for Na^+ by these forms appears to be almost unique among bacteria so far investigated. In the case of only one representative of any other group of bacteria has evidence for a Na^+ requirement been obtained. The halophile *Pseudomonas salinaria* has recently been shown to require Na^+ for growth (Brown and Gibbons, '55).

In this study the quantitative requirements for Na^+ and K^+ and the specificity of these requirements have been determined for three marine bacteria. Information regarding the intracellular concentrations of the ions relative to their concentration in the medium has been obtained. The findings indicate that there is a marked similarity between marine bacterial cells and animal cells as regards both the relationship between intracellular and extracellular Na^+ and K^+ levels and the quantitative requirements of both types of cells for these ions for growth.

MATERIAL AND METHODS

Cultures. The sources of the organisms and the general methods used in their study have been described (MacLeod et al., '54, '56). Three of the organisms studied previously were

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selected for this work. They have been tentatively identified as B_9, a *Flavobacterium;* B_{10}, a *Corynebacterium,* and B_{16}, a *Mycoplana.*[1]

Assay procedures. The composition of the basal and inoculum media, the preparation of the inocula, and the assay procedures used have been described (MacLeod and Onofrey, '56).

Analytical methods. Analyses for Na^+ and K^+ were made with a flame photometer attachment for a Beckman DU spectrophotometer equipped with a photomultiplier. Na^+ and K^+ standards were prepared containing ions at levels similar to those present in the solutions to be analyzed. Analyses were carried out directly on the untreated media and on dry-ashed cell preparations.

RESULTS

Response to Na^+

The bacteria investigated would not grow in a medium prepared with artificial sea water if Na^+ was omitted (MacLeod and Onofrey, '56). Growth could not be restored if Na^+ was replaced by equimolar concentrations of either K^+ or glycerol. This suggested a specific function for Na^+ in the growth of the organisms apart from any role it might play in maintaining proper osmotic pressure relations for the cells.

The response of the organisms to various levels of Na^+ in a Na^+-deficient medium was determined. The results obtained with organism B_{16} are presented in table 1. It is evident that the level of Na^+ in the medium affected both the rate and extent of growth. Both the maximum rate and extent of growth was achieved with 2 millimoles of Na^+ per 10 ml of medium. This level of Na^+ is slightly less than half that found in sea water. Below the 2 millimole level both the rate and the extent of growth were roughly proportional to the amount of Na^+ added.

[1] A recent study of the classification of these organisms conducted by Dr. Einar Leifson has shown that B_{10} is a species of the genus *Pseudomonas* and B_{16} belongs to either the genus *Pseudomonas* or *Spirillum.* We are deeply indebted to Dr. Leifson for undertaking this investigation.

Growth occurred at the 0.3 millimole level of Na^+ after a 10-day incubation period though not at still lower levels by 21 days. Once cultures had ceased growing they began to autolyze rapidly. Though not shown here, separate experiments have revealed that a level of 8 millimole of Na^+ per 10 ml of medium inhibited the growth of all three organisms. This amount of Na^+ is supplied in a medium containing approximately 5 per cent NaCl. Organisms B_9 and B_{10} responded like B_{16} to Na^+ except that in the case of B_9 there was almost no tendency for the cultures to autolyze after growth had ceased.

TABLE 1

The response of marine bacterium B-16 to Na^+ in a Na^+ deficient medium

Na^+ [1]	INCUBATION TIME (HR.)			
Millimoles/10 ml	*48*	*72*	*120*	*312*
	Percent incident light transmitted [2]			
0.0	100	100	100	100
0.1	100	100	100	100
0.3	100	100	100	44
0.5	100	100	89	—
0.7	98	39	35	—
1.0	86	39	46	—
1.3	34	30	60	—
2.0	26	30	52	—
3.0	25	34	51	—

[1] Added as NaCl.

[2] Evelyn colorimeter readings 660 mμ filter. Uninoculated medium = 100.

Since the level of Na^+ required for optimum growth is considerably higher than that of other inorganic ions involved in bacterial nutrition (MacLeod and Snell, '47), it seemed not unlikely that at least part of the function of Na^+ might be to maintain an appropriate osmotic pressure in the medium. To test this possibility, the response of the three organisms to Na^+ was determined in the presence and absence of sucrose, since sucrose has a low rate of penetration into cells, at least in some biological systems (Collander and Börland, '33) and is not metabolized by cell suspensions of organism B_{16}. It was found that all of the organisms grew at slightly lower

levels of Na^+ in the presence of sucrose than in its absence, the sparing action being more pronounced after short incubation periods. It seemed evident from the results, however, that the maintenance of a high osmotic pressure in the medium was by no means the primary function of Na^+ in the growth of the cells.

Specificity of the Na^+ requirement

The ability of Li^+, K^+, Rb^+ and Cs^+ to replace or spare the requirement of the three organisms for Na^+ was determined.

TABLE 2

Response of marine bacterium B-10 to Na^+ in the presence and absence of K^+ added in excess of growth requirements

Na^+	Incubation time (hr.)			
	68		241	
Millimoles/10 ml	K^+ added (*millimoles/10 ml*)			
	0	1	0	1
	Percent incident light transmitted [1]			
0.0	100	100	100	100
0.1	100	100	100	100
0.3	100	100	85	67
0.5	100	70	50	73
1.0	99	43	79	69
1.3	46	43	78	73
1.5	37	15	71	42
2.0	20	14	67	32

[1] See table 1.

None of the ions permitted growth when added in place of Na^+ to the deficient medium. When the response of each organism to Na^+ in the presence and absence of 1 millimole of each of the related ions was compared Li^+, K^+, and to a lesser extent Rb^+ had sparing actions for all three organisms after short incubation periods which were barely detectable after longer ones. Cs^+, on the other hand, was somewhat inhibitory at these concentrations. The nature of the sparing actions obtained is illustrated in table 2 where the effect of K^+ on the

response of B_{10} to Na^+ is shown. It is evident that after 68 hr of incubation between 1.0 and 1.3 millimoles of Na^+ per 10 ml was required for growth in the absence of K^+ and between 0.3 and 0.5 millimoles in its presence. After 241 hr there was little difference in the Na^+ requirement in the presence and absence of K^+. This sparing action of K^+ on the Na^+ requirement was quite similar to that obtained with sucrose. The decrease in turbidity of some of the cultures on longer incubation was due to autolysis occurring after growth had ceased.

The possibility that the inhibitory action of Cs^+ might be due to an ability of the ion to interfere with the utilization of Na^+ was considered. No evidence of an antagonism between Na^+ and Cs^+ could be demonstrated for any of the organisms.

Response to K^+

When the quantitative requirements of the organisms for K^+ were determined it was found that only about one one-hundredth as much K^+ as Na^+ was needed. As in the case of Na^+, the amount of K^+ in the medium affected not only the extent but also the rate of growth of the organisms. These observations are illustrated in the response of organism B_{10} to K^+ after three periods of incubation. (Table 3, response in the absence of Cs^+). It is evident that although maximum growth of the organism was obtained in 48 hr in the presence of sufficient K^+, extensive additional growth occurred at suboptimal levels on longer incubation. Organisms B_9 and B_{16} responded to K^+ in the same way as B_{10}.

Neither Li^+ nor Cs^+ were able to replace K^+ for the growth of the organisms. When Rb^+ was tested it was found to be capable of replacing K^+ but only when added in high concentrations. When the Rb^+ salt used was analyzed with the aid of a flame spectrophotometer, it was found that the response to Rb^+ could be accounted for quantitatively by the amount of K^+ present as a contaminant. This was confirmed by removing all but traces of K^+ from the Rb^+ salt by ion exchange chromatography using Amberlite IR-120 and a pro-

cedure similar to that described by Cohn and Kohn '48). The response to the purified Rb^+ salt was again proportional to the K^+ present.

Cs^+ and Li^+ failed to replace K^+ and also did not promote growth in the presence of suboptimal levels of the ion. The apparent sparing action of Rb^+ could be accounted for again by the level of K^+ present as a contaminant in the Rb^+ salt.

TABLE 3

Response of marine bacterium B-10 to K^+ in the presence and absence of Cs^+

K^+	Cs^+ (millimoles/10 ml)								
	0.0			0.3			0.5		
Micromoles/10 ml	Time (hr.)								
	48	96	144	48	96	144	48	96	144
	Percent incident light transmitted [1]								
0	100	100	100	100	100	100	100	100	100
1	—	—	100	—	—	—	—	—	—
1.5	100	100	98	—	—	—	—	—	—
2	98	97	58	—	—	—	—	—	—
3	72	59	71	—	—	100	—	—	—
4	38	77	87	—	100	89	—	—	100
10	9	28	37	—	88	15	—	—	98
30	5	19	26	—	4	9	—	100	7
100	6	21	29	100	5	16	—	19	20
1000	14	36	45	12	27	33	100	17	18

[1] See table 1.

Ion antagonism

When Li^+, Rb^+ and Cs^+ were tested to determine their toxicity for the three organisms, Cs^+ was the only ion significantly inhibitory below a level of 2 millimoles per flask, and then only for organisms B_9 and B_{10}. Evidence showed that at least a part of the toxicity of Cs^+ was due to its ability to interfere with the response of the organisms to K^+. This is evident from the results in table 3 where it can be seen that considerably more K^+ was required to obtain growth in 48 hr in the presence of 0.3 millimoles of Cs^+ than in its absence. At the higher Cs^+ level tested no growth was apparent in 48 hr even at the

highest level of K^+ tested though growth did occur on longer incubation. It would thus appear that Cs^+ interfered with the ability of K^+ to affect both the rate and the extent of growth of the organism.

Ion uptake by growing cultures

Because of the size and specificity of the Na^+ requirement, further information concerning the relation of the ion to the growth of the cells was sought. Cultures were grown in media containing suboptimal levels of Na^+ until the amount

TABLE 4

Uptake of Na^+ and K^+ from the medium by cells of marine bacterium B-10 during growth

TURBIDITY [1]	Na^+ in medium		UPTAKE	TURBIDITY [1]	K^+ in medium		UPTAKE
	Before growth	After growth [2]			Before growth	After growth	
	mg/10 ml				*μg/10 ml*		
62	5.7	5.6	+.1	96	118	113	+ 5
57	9.0	9.1	—.1	91	141	131	+ 10
50	11.9	12.0	—.1	75	136	121	+ 15
4	23.7	23.5	+.2	42	162	139	+ 23
				12	300	182	+ 118

[1] Percent of incident light transmitted.

[2] Cultures were incubated until growth ceased.

of growth permitted by the level of Na^+ present was attained. The cells were removed by centrifugation and the Na^+ concentration in the supernatant medium compared with that in an uninoculated control tube. The results, table 4, indicate that no net uptake of Na^+ by the cells could be demonstrated by this method. When the same technique was applied to cultures grown in the presence of suboptimal levels of K^+, evidence of uptake of K^+ by the cells was obtained, though again considerable quantities of the ion remained in the medium after growth had ceased, (table 4). These results indicate that when growth is limited by the level of either Na^+ or K^+ in the medium, the limitation of growth is not due to the exhaustion of the supply of either ion by the cells.

Although no net uptake of Na^+ by the cells could be demonstrated by analysis of the supernatant medium, it was still possible that there was Na^+ present in the cells. Even if Na^+ were concentrated to some extent by the cells, the amount of Na^+ removed from the medium relative to the total present would still be so small that the error inherent in the analytical method used would not permit the detection of the uptake if the analysis was applied to the medium. To determine whether or not Na^+ and K^+ were taken up by the cells, and if so what the relative concentrations of the ions in the cells and in the medium might be, the following experiments were performed. Cells were harvested from a medium by centrifuging at 20,000 $\times$ G in a Servall angle-head centrifuge. The cell pad obtained was drained, weighed, ashed and analyzed for its content of Na^+ and K^+. The supernatant medium was diluted appropriately and its Na^+ and K^+ content determined in the flame photometer directly. In order to express the concentrations of both fractions in terms of weight per unit volume, the assumption was made that the specific gravity of the cell pad was one. Since the specific gravity of the cells is slightly greater than one, the concentrations in the cell pads were actually slightly higher than the results obtained would indicate. The ratio of the concentration of each ion in the cell pad to its concentration in the supernatant medium was calculated. From the ratios obtained one can conclude whether the concentration of the ion in the cells is equal to, less than, or greater than that in the medium depending on whether the ratio is equal to, less than, or greater than one (Robertson et al., '55). The ratios obtained when organism B_{16} was grown in the presence of various levels of Na^+ and K^+ are shown in table 5. In each case a crop of cells harvested from 200 ml of medium was analyzed for its Na^+ and K^+ content and each result reported represents the average of five determinations. The organism was grown at three different levels of Na^+, while K^+ was maintained constant at the level occurring in sea water. It can be seen that when the Na^+ level was 0.64 millimoles/10 ml, which is suboptimal for maximum rate and

extent of growth, the Na^+ level in the cells was somewhat less than in the medium. When the Na^+ level reached 1.38 millimoles, which is close to the optimum for growth, the Na^+ level in the cells and in the medium was approximately the same. At a Na^+ concentration of 4.19 millimoles per 10 ml which is approximately the level of Na^+ in sea water, there was less Na^+ in the cells than in the medium. The corresponding K^+ ratios show that in all cases K^+ was at a higher concentration in the cells than in the medium and as the Na^+ level in the medium increased to the level of Na^+ in sea water, the inter-

TABLE 5

The effect of medium levels of Na^+ and K^+ on the ratio of the concentration of each ion in the cell preparation to its concentration in the growth medium for organism B-16

MEDIUM LEVELS [1]		RATIOS OBTAINED [2]	
Na^+	K^+	Na^+	K^+
Millimoles/10 ml			
0.64	0.097	0.945 ± 0.015	1.333 ± 0.004
1.38	0.104	0.978 ± 0.019	1.367 ± 0.022
4.19	0.099	0.817 ± 0.015	1.792 ± 0.088
1.41	2.05	0.812 ± 0.005	1.031 ± 0.012

[1] The medium levels indicated are the starting levels in the uninoculated media.
[2] The ratios obtained for each ion = concentration of the ion in the cell preparation/concentration of the ion in the medium after growth had ceased.

nal K^+ concentration increased. When the K^+ concentration was increased 20-fold to 2.05 millimoles per 10 ml and the Na^+ level maintained at the optimum level for growth, the K^+ content of the cells and medium was very nearly the same while the Na^+ level in the cells dropped from that occurring when the cells grew in a medium containing the same Na^+ level but a lower concentration of K^+. The medium having Na^+ at 4.19 millimoles and K^+ at 0.1 millimoles per 10 ml contained these ions at approximately the levels at which they occur in sea water. As can be seen, cells from this medium contained less Na^+ and more K^+ than the medium.

DISCUSSION

The ratios of the major salts to each other and usually their total concentrations also are strikingly similar in sea water and in the body fluids of animals (Pantin, '31, Dakin, '35). Animal cells contain more K^+ and less Na^+ than is present in the body fluids in which they are bathed. The results reported here indicate that when Na^+ and K^+ are present in the medium at the concentrations occurring in sea water, cells of the marine bacteria studied also contain more K^+ and less Na^+ than is present in the fluid surrounding them. It is thus apparent that mechanisms for the selective elimination of Na^+ and uptake of K^+ occur in marine bacteria as well as in the cells of higher forms of life. Since bacteria are believed to have arisen at an early period in the evolution of life (Oparin and Morgulis, '38), such mechanisms must thus have developed at a very early stage.

Another striking similarity between marine bacterial cells and animal cells is evident when the requirements of both for Na^+ and K^+ are compared. In a study of the salt requirements of two types of mammalian cells for optimum growth in tissue culture, Eagle ('56) has shown the Na^+ requirement to be 110–140 millimoles (when Na^+ supplied by the buffer is included) and the K^+ to be 1–10 millimoles. The corresponding requirements for marine bacteria expressed in the same units are 150–300 millimoles for Na^+ and 1–3 millimoles for K^+, depending on the organism.

As the need for Na^+ distinguishes marine bacteria from all other bacteria so far investigated with the exception of the halophile *Pseudomonas salinaria* (Brown and Gibbons, '55), the possible relation of marine bacteria to these other organisms is of interest. Halophilic bacteria belong to a rather ill-defined group, some members of which require 15–20 per cent sodium choloride in the medium for growth whereas others, described as moderate halophiles, grow over a much wider range of salt concentration (1–20 per cent) (Baxter and Gibbons, '54). Since the marine bacteria investigated here are

inhibited by the presence of as little as 5 percent NaCl in the medium, it would appear that they can be most readily distinguished from halophiles by their lack of tolerance for high concentrations of salt in the growth medium. Since life is believed to have originated in the sea it is not unlikely that terrestial bacteria are marine bacteria which have become adapted to growth on land due to the development of an ability to grow without Na^+ while halophiles are marine bacteria which have developed the capacity to tolerate high concentrations of salt.

The requirement of the organisms for Na^+ and K^+ appears to be highly specific. The slight ability of the various alkali-metal ions and sucrose to improve the rate but not the extent of growth of the organisms in the presence of suboptimal concentrations of Na^+ may be a measure of the degree to which Na^+ functions to maintain the proper osmotic pressure relations for the cells. The inability of Rb^+ to substitute for K^+ in whole or in part for these marine bacteria contrasts with the capacity of this ion to both spare and replace K^+ in the nutrition and metabolism of various lactic acid bacteria. The antagonism between K^+ and Cs^+ demonstrated here, however, has been observed with the latter group of organisms (MacLeod and Snell, '48).

That mechanisms for maintaining K^+ at a higher concentration in the cells than in the medium are operative in marine bacteria is evident not only from the ratios in table 5 but also from washing experiments. In the latter, Na^+ but not K^+ was found to be readily removed from the cells by washing with cold sucrose solutions. Similar findings have been recorded for *E. coli* (Cowie et al., '49). In view of the ability of the cells to concentrate K^+ it was of interest to find that at levels of K^+ suboptimal for growth considerable amounts of K^+ remained in the medium after growth had ceased. To determine how general this phenomenon was, cells of *Lactobacillus arabinosus* were grown in media containing less K^+ than was required for maximum growth (MacLeod and Snell, '48). Analysis of the supernatant medium after growth had ceased revealed

results essentially the same as those obtained with the marine bacteria.

SUMMARY

For the three marine bacteria investigated, the levels of Na^+ and K^+ in the medium were found to affect both the rate and the extent of growth. Only one one-hundredth as much K^+ as Na^+ was required. Neither Li^+, Rb^+ nor Cs^+ permitted growth of any of the organisms when added in place of either Na^+ or K^+. Cs^+ was found to be inhibitory for two of the organisms tested and evidence for antagonism between K^+ and Cs^+ was obtained.

When growth of the organisms was limited by the level of either Na^+ or K^+ in the medium, analysis of the supernatant medium after growth had ceased revealed that considerably more ions remained in the medium than were taken up by the cells.

At suboptimum and optimum concentrations of Na^+ for growth, intracellular and extracellular Na^+ concentrations were nearly the same, while at the level of Na^+ in sea water the extracellular concentration exceeded the intracellular. When K^+ was present in the medium at the level found in sea water the cells contained more K^+ per unit volume than the medium.

Attention has been drawn to the similarity between marine bacterial cells and animal cells as regards both the relationship between intracellular and extracellular Na^+ and K^+ levels and the quantitative requirements of both types of cells for these ions for growth. It has been suggested that both terrestrial bacteria and halophilic bacteria have evolved from marine bacteria, the former by developing an ability to grow without Na^+ and the latter by becoming adapted to growth in the presence of high concentrations of salt.

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BIOCHEMISTRY OF FISHES[1,2]

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Since this subject was last reviewed (1) articles summarizing the advances which have been made in various phases of the biochemistry of fish have appeared from time to time. Recent publications have dealt with physiology (2), marine biochromes (2, 3), assessment of nutritive value and analytical data (2, 4 to 7), and marine oil research (6 to 10). These general topics have therefore been excluded from the present review, which has been devoted to a limited number of general subjects of fairly immediate interest. With the exception of the section on marine sterols, a subject in which research has been almost exclusively confined to invertebrates, only literature dealing with true fish (Pisces) has been considered.

Proteins

A number of important contributions to our knowledge of fish muscle proteins have been made. The general fractionation methods employed (11 to 15) have resulted in better separation of the proteins than was obtained by earlier investigators (16, 17, 18), largely because of the availability of the techniques of electrophoresis and ultracentrifugation.

The proteins extractable by cold neutral salt solutions of fairly high ionic strength include myosin, actin, and tropomyosin. The conditions for extraction employed by different investigators have not been consistent so that the results are not strictly comparable; post-mortem age, degree of comminution, pH, extraction time, and ionic strength of extractant solution may all have affected results. Salt (e.g., NaCl, KCl, LiCl) solutions of μ 0.5 to 1.0 and pH 6.0, and preferably between pH 7.0 and 7.5, have usually proved satisfactory (11, 13, 14) for extraction. Solutions of μ 0.3 to 0.45 have proven successful with some muscles (12, 15). Most investigators who have studied the protein fraction extractable under the above conditions, which is precipitated by adequate dilution with water ($\mu < 0.25$), have

[1] The survey of the literature pertaining to this review was completed in October, 1957.

[2] The following abbreviations are used in this chapter: ADP for adenosine diphosphate; AMP for adenosine monophosphate; ATP for adenosine triphosphate; ATPase for adenosine triphophatase; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; DR-1-P for deoxyribose-1-phosphate; GDP for guanosine diphosphate; GMP for guanosine monophosphate; GTP for guanosine triphosphate; IMP for inosine monophosphate; PAB for *p*-aminobenzoic acid; PGA for pteroylglutamic acid; RNA for ribonucleic acid; UDP for uridine diphosphate; UMP for uridine monophosphate; and UTP for uridine triphosphate.

[3] The assistance of Dr. D. R Idler in preparing the section on Sterols of Marine Invertebrates is greatly appreciated.

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517

worked with more or less pure actomyosin. The ultracentrifuge pattern of carp actomyosin extracted at pH 7.0 showed a single sharp peak, and in its physical properties it did not differ in some respects from rabbit muscle actomyosin (11, 12). Data on the physical properties of actomyosin prepared from other fishes are lacking.

Myosins were prepared from carp (11) and from cod (19) muscles by different procedures, but were not homogeneous by ultracentrifugal analyses, probably due to the presence of contaminating components. When carp muscle actomyosin was subjected to ultracentrifugal analysis in the presence of ATP, myosin, with a sedimentation constant of 6.55, which is not greatly different from that of rabbit myosin, separated (11, 12). It has been found that the amount and kinds of stroma proteins in both cod and carp muscles make it more difficult to prevent simultaneous extraction of actin and myosin (as actomyosin) than is the case with rabbit muscles. There has also been found to be a parallel between the rate of extraction of actin from minced fish muscles and the amount of stroma proteins present (20).

Tropomyosins have been prepared from fish muscles by solvent extraction (12, 21, 22), and by ammonium sulfate fractionation and electrophoresis (22). Several tropomyosins with somewhat different physical properties, including crystalline tropomyosin and nucleotropomyosin, have been prepared from carp muscles and their physical properties described (11, 12, 21, 22, 23). G actin has been prepared from cod muscle, and in the presence of salts this polymerized to viscous F actin (19). F actin has also been demonstrated in carp muscles (11).

Recent investigations have shown that the rate at which myosin in intact frozen cod muscles denatures during prolonged frozen storage (e.g., 390 days at −30°C.) is influenced markedly by the rate at which the tissues have been frozen. There appears to be a rather critical freezing period above or below which myosin denaturation, as measured by its tendency to become insoluble in cold neutral 0.85 *M* NaCl solutions, is comparatively slight (24). It has long been considered that the organoleptically detectable toughness of fish muscles which occurs on prolonged frozen storage, particularly at elevated temperatures (e.g., 10°C.), is directly connected with denaturation of myosin as measured by its insolubility in neutral salt solutions of high ionic strength (25). However, recent investigations have shown that, under certain conditions, the former alteration occurs with only an extremely slight decrease in myosin solubility (24, 26).

The separation of fish muscle myocommata from the myotomes offers considerable promise in facilitating determinations of the myosin content of the latter, and the effect of age, sexual development and season on this (27). Changes in viscosity and sulfhydryl groups in stored frozen fish muscles have been measured (28).

Fish muscle proteins extractable by salt solutions of low ionic strength ($\mu < 0.15$) include those which have been named myogens, myoalbumins and globulin X. They normally account for about 21 to 22 per cent of the total

muscle protein of teleost fishes (11 to 14, 29, 30). The low ionic strength extractable protein fraction obtained from a number of marine and fresh-water fish, including teleosts and elasmobranchs, has been subjected to electrophoresis both by the moving boundary and zone methods (11, 12, 15, 31, 32, 33, 34, 35). There appear to be from seven to ten different protein fractions in these extracts as determined by the above methods, and, where comparisons have been made, both methods yield somewhat similar results. It seems to be possible to divide these proteins into three main groups, and there is a fairly general similarity in the electrophoretic patterns obtained, but there are distinct species differences. Scanty available data do not indicate that there is any important difference between the electrophoretic patterns of low ionic strength extracts of carp white and red lateral muscles (12). A crystalline myogen with a molecular weight of about 67,000 has been isolated from carp muscles, and it appeared to be homogeneous by ultracentrifugal analysis (36), but gave two peaks by electrophoresis (37). The electrophoretic inhomogeneity has been shown to be due to a small amount of a contaminating protein with a comparatively low molecular weight. A very similar protein, but one with a different sedimentation constant, has been isolated from flounder muscles (38).

Red lateral muscles of certain fish have been shown to be rich in both cytochrome and myoglobin (39), and crystalline myoglobins have been isolated from several species of fish (40, 41, 42). Red lateral muscles of fish have been reported as being rich in cytochrome-*c* (39), and cytochrome-*c* isolated from tuna red muscle has been reported to differ somewhat spectroscopically from that of horse heart muscle (43). Crystalline cytochrome-*c* preparations isolated from the heart muscles of tuna species, and from bonito, appear to be practically identical spectroscopically, but differ somewhat from that of crystalline beef heart muscle cytochrome-*c* (44). The haemoglobin content of the blood of migratory fish has been reported to be greater than that of less active fish (45). The haemoglobin content of fish blood is very variable, e.g., 2.0 to 11.8 gm. per 100 ml. (46, 47). However, this is to be expected in view of the findings that the amount of respiratory pigment and erythrocytes depends on the oxygen requirements of fish (48).

A number of different methods of preparation of the protamines have been recorded. These preparations have not been absolutely homogeneous when examined by the techniques of electrophoresis, ultracentrifugation or counter current distribution (50, 51, 52). Clupein, salmine, and sturine are of relatively simple composition and contain six to 10 different amino acids. Molecular weights of from 3,800 to 10,000 have been reported for different protamine preparations (51 to 54). The arginine content of clupein has recently been reported as about 88 per cent (49). Recent physical and chemical investigations with various protamines indicate that they are composed of a number of different proteins of different molecular weights and amino acid composition (51, 53, 54). Considerable progress has been made in determining the N- and C- terminal amino acids in certain of the protamines, and

also the amino acid sequence (51 to 57). It has been indicated that about one mole of nucleic acid combines with 100 of protamine, and that a large number of different nucleoprotamines could exist (51).

The only connective tissue protein of fish which has been studied to any extent is collagen, and much of the early information regarding this has been reviewed (58). X-ray studies have indicated that teleost collagen, ichthyocol, differs somewhat from the elasmobranch collagen, elastoidin. Many differences have been found between the collagen obtained from codfish skins and bovine collagen (59, 60). The total amino acid composition of a number of fish collagens has been determined, and a very similar distribution found. However, there were notable differences, especially with respect to a lower hydroxyproline and proline content, from mammalian collagen (61). About one third of the total nitrogen of whole herring appears to exist as gelatin nitrogen, which is divisible into three fractions as based on solubility criteria. Significant seasonal changes in the proportions of these fractions have been found (62). The essential amino acid composition of whole herring scales has been determined (63), and the following composition of pilchard scales has been reported: mineral matter, 56 per cent; ichthylepidin, 21 per cent (molecular weight about 200,000); and gelatin, 23 per cent (64). A pseudokeratin, designated ichthulokeratin, has been found in salmon eggs. It has a histidine:lysine:arginine ratio of 1:3:4.5, and is digested by pepsin and not by trypsin (65). The amino acids occurring in acid hydrolysates of fish lens proteins have been reported (66). The rather limited number of studies which have been carried out on fish plasma proteins indicate that there are great variations between both closely related and unrelated species, and that there is little or no protein which is similar electrophoretically to the gamma globulin of the mammals (32, 67, 68, 69).

Nonprotein Nitrogen

This fraction is said to account for from 9.2 to 18.3 per cent of the total nitrogen in teleost fishes and from 33 to 38.6 per cent in elasmobranch fishes. The compounds occurring in this fraction have been grouped as follows: (*a*) volatile bases (ammonia, mono- di- and trimethylamine), a group which is of minor significance in muscles of living fish; (*b*) trimethylammonium bases (trimethylamineoxide, betaines); (*c*) guanidine derivatives (creatine and arginine); (*d*) imidazole or glyoxaline derivatives (histidine, carnosine, and anserine); (*e*) miscellaneous (urea, amino acids, and purine derivatives) (70). The occurrence of these soluble nitrogenous compounds in the muscles of fish has been the subject of fairly extensive investigation.

An alcohol extract of haddock muscle was subjected to displacement chromatography and the amino acids and related compounds in the various fractions were identified by paper chromatography. The following substances were found: leucine, glycine, alanine, glutamic acid, serine, creatine, creatinine, β-alanine, trimethylamineoxide, anserine (comparatively large amounts), valine, threonine, choline, aspartic acid, cysteine, histidine,

methylhistidine, lysine, arginine (moderate amounts), and traces of tyrosine and phenylalanine. Ammonia, mono- and dimethylamines were present in only trace amounts and carnosine was absent. Crystalline anserine was isolated and identified (71). Subsequent investigations of nine species of gadoid fish showed that the content of soluble nitrogenous compounds in the muscle was rather similar to that of the haddock. However, some interesting differences were noted. Thus, betaine and sarcosine were present only in elasmobranch fish, and anserine and methylhistidine in the gadoid fish. Traces of glutathione were found. Crystalline betaine was prepared from skate muscle (72). Further work (73) showed that flat fish yielded smaller amounts of trimethylamineoxide and anserine than did gadoid fish, and that 1-methylhistidine appeared to be absent. Pelagic fish (tuna and mackerel) were unique in that their muscles contained comparatively large amounts of histidine but no anserine or carnosine. Several unidentified compounds were demonstrated in the above studies. This work indicated that there is probably a definite generic pattern in the distribution of certain of the soluble nitrogenous compounds in fish muscles.

Taurine, which was not detected by the above investigators due to the ion exchange resin technique employed, was subsequently demonstrated in comparatively high concentrations in extracts of cod muscle. It was closely similar in concentration to trimethylamineoxide and creatine which occur in fish muscles (74). The following concentrations of free amino acids (mg. per 100 gm. wet weight) have been reported in cod muscles (75 per cent ethanol extracts used): taurine 300, glycine 21, alanine 15, glutamic acid 8, 1-methylhistidine 6.7, leucine 7.1, valine 3.5, β-alanine 1.2, aspartic acid 1.9, serine 1.4, lysine 1.9, cysteic acid 1.2, histidine less than 0.5, and traces of proline, tyrosine, phenylalanine, and threonine. Interesting seasonal variations in the concentrations of certain of these extractives were observed. Thus taurine reached a peak in May to July (300 to 450 mg.) and was at a minimum in December to February (100 to 150 mg.). The lysine content, which was 0.5 mg. throughout most of the year, rose to 12 to 15 mg. in January and February during the spawning period (75). Recently an increase in the lysine content of both male and female sturgeon muscle during spawning migration has been reported (76). It has been shown that, with few exceptions, the free amino acid content of aseptically excised fish muscles does not increase appreciably in 25 days at 0°C. The glutamic acid content increased from 10 to 29 mg. per 100 gm., the β-alanine from 2.5 to 66 mg. per 100 gm. and the 1-methylhistidine content from 6.2 to 102 mg. per 100 gm. Comparatively slight decreases in the alanine and lysine content were observed (77).

The distribution of the imidazole bases histidine, 1-methylhistidine, carnosine, and anserine, in the flesh of a large number of North Pacific fish marine mammals, and invertebrates, has been studied. Quantities of these bases varied from approximately 2 to 56.8 μg. per gm. Interesting species differences in the distribution were recorded (78). Paper electrophoresis of

aqueous extracts of fresh crawfish and mussels have produced fraction samples which are specific for each species, and the electrophoretic patterns change during prolonged storage up to 40 days at 0°C. (79). It has been reported that the muscles of crustacea contain a greater content of free amino acids than do those of the fishes (80). A peptide was isolated from the muscles of a number of sea fish that did not occur in the muscles of fresh-water fish. This peptide, on acid hydrolysis, yielded glutamic acid, aspartic acid, glycine or serine, α-alanine and very little threonine (81).

The distribution of urea, trimethylamineoxide, and ammonia in a large number of species of fish is now known (70, 82). It has been suggested that the desirable flavour of a number of different molluscs and fishes is due to the high content of free amino acids and more particularly to the monoamino acids (82 to 85), and in case of certain pelagic fish, to the histidine (86) present in their muscles.

Nucleic Acids and Related Compounds

The discovery of the carp muscle protein, nucleotropomyosin, marked the first clear-cut demonstration of the presence of RNA in fish muscles (22). More recently it has been demonstrated that RNA is present in concentrations of from 42–142 μg. per gm. in the muscles of Pacific Ocean fish (87). RNA has been prepared in comparatively pure state from muscles of lingcod and sockeye salmon (87), and the distribution of the bases adenine, guanine, cytosine and uracil (molar ratio approximately 1:2:1:1.25) has been found to differ somewhat from that of the RNA of carp nucleotropomyosin (molar ratio 1:2.1:1.85:1.10) (22). The ratio of purines to pyrimidines was somewhat higher, and of nitrogen to phosphorus somewhat lower, than that recorded for yeast RNA (87). The enzymic degradation of RNA by fish muscle enzymes is being studied actively (see section on Enzymes).

Initial attempts to determine DNA in fish muscle aqueous extracts met with failure (22, 88), probably due to the comparatively low concentrations in which it occurs, and the fact that cell damage is apparently required to liberate it from the muscle cells (89). The highest concentration of DNA phosphorus obtained from cod muscle press juice was 0.628 mg. per 100 ml. (90). The DNA content of the expressible fluids of fish muscles appears to yield useful information regarding cell damage which occurs during freezing (90, 91). The muscles of six species of Pacific coast fish contained from approximately .02 to 0.25 mg. of DNA phosphorus per 100 gm. of wet muscle (87). These values are considerably lower than those recorded for mammalian muscles where concentrations from 4.3 to 9.6 mg. of DNA phosphorus per 100 gm. have been reported (92). Muscles of Pacific herring have been found to contain between 1.87 and 3.51 mg. of DNA per 100 gm., and between 10.4 and 26.5 mg. per 100 gm. of RNA (93). The following molar ratio of bases in highly polymerized herring roe DNA has been recorded: adenine 1.0, guanine 0.55, cytosine 0.70, thymine 1.01. Chemical degradation studies of this product suggested that there are regions where at least three pyrimidine nucleo-

tides are linked together (94). It has been reported that a single whole or cytolized defatted trout sperm contains 2.45×10^{-9} mg. of DNA and 1.55×10^{-9} mg. of arginine (95).

Several investigators have studied the nucleotides of fish tissues, and more particularly of the muscle. An investigation of the muscles of five Pacific coast fish which were obtained in considerably different physiological condition indicated that the sum of the ATP and ADP in these muscles varies from 0.27 to 3.72 μM per gm. expressed as ATP. The lower values were obtained particularly in fish muscles which were stored for two days at 0°C., or had been obtained from fish which had struggled considerably (96). Rather similar values have been obtained with pike and tench (97). The nucleotide content of skeletal muscle of the cod has been investigated in detail (98). For rested fish the following values were obtained (μM per gm.): ATP, 5.34; ADP, 0.576; AMP, 0.069; DPN, 0.107; IMP, 1.26. Decreases in both ATP and ADP were noted in exhausted fish or in fish which had just passed out of rigor. In this study comparatively large amounts of IMP accumulated post mortem due to the activity of a deaminase. Small amounts of GTP, UTP, CTP and "ADX" were identified among a number of minor constituents. The presence of a nucleotide which contains a purine substituted in the C-6 position, and which has an absorption spectrum similar to that of adenylsuccinic acid, has been demonstrated in cod livers (99). Salmon livers have been found to contain DPN, AMP, GMP, UMP, IMP, ADP, UDP, GDP, ATP, uric acid, ADP-acetylglucosamine (100), and a new adenylsuccinic acid derivative containing sulfate and a peptide (101).

It has been stated that the RNA content of salmon eggs is greatest during embryonic growth and that during critical periods the RNA values are low (102). It has also been reported that in the development of ovogonia to ovocytes the RNA in the cytoplasm increases and declines after the appearance of yolk granules (103). Ripe salmon testes contain 7.5 per cent of sodium deoxyribonucleate and a method whereby this has been isolated almost quantitatively has been described. Salmine can be prepared readily as a by-product of this method (104).

Phospholipides

The literature concerning the very plentiful triglycerides of fish tissues has been much more extensive than that relating to the much less plentiful phospholipides. The content in fish tissues of phospholipides is comparatively low as the following values indicate (per cent of wet weight): flesh 0.1 to 0.85: (105 to 109); liver 0.16 to 0.49 (106, 110, 111); eggs 0.4 to 4.0 (114, 115); intestines 0.41 to 1.0 (106, 112); brains about 4 per cent (116, 117, 118), and spinal cord about 6 per cent (119).

Until recently the subdivision of the total phospholipide fraction of fish tissues has proved difficult due to the inadequacy of the fractionation procedures available. Thus attempts made prior to about 1952 to divide the phospholipides of fish organs into fractions such as lecithin, cephalin and

sphingomyelin have yielded results which can only be regarded as rough approximations (120). Practically all recent work on fish muscle phospholipides has been carried out with cod and haddock in which the total lipide of the flesh is relatively low and practically free of triglycerides. Thus both cod and haddock muscle contain approximately the same amount of total lipides, namely 0.6 per cent. The composition of this fraction in haddock and cod flesh respectively has been recorded as follows (per cent wet weight): lecithin 42.8, 35; unidentified lipides 15.2, 21; waxes and alcohols 10.5, 13; free cholesterol 6.1, 8; free fatty acids 6.1, 6.0; phosphatidyl ethanolamine 5.4, 8.0; inositol lipides 4.4, 2.0; cholesterol esters 3.4, 5.0; triglycerides 2.4, 3.0; hydrocarbons demonstrated in the earlier work are probably contaminants (108, 121). Quantitative extraction has involved use of a series of solvents (122), removal of impurities from the extract so obtained (108, 109), and fractionation of the purified extracts by means of counter-current distribution between aqueous ethanol and light petroleum. Resulting fractions, or hydrolysates obtained from them, have been analyzed for all known constituents. The sum of the content of fatty acids, total unsaponifiable matter, free and esterified cholesterol, higher aliphatic aldehydes, glycerophosphoric acid, glycerol, inositol, choline, ethanolamine, and serine has been used to indicate the composition of a particular fraction, and thus of its total composition. The results have indicated the great complexity of the mixture of lipides in these tissues, and also the presence of new and uncharacterized lipides. The following values have been reported for the phospholipides of the flesh of the cod and haddock respectively (percentage of total phospholipides): lecithin 54, 62; phosphatidyl ethanolamine 11, 8; phosphatidyl serine, absent; inositol phosphatides 3, 6; plasmalogens, present, 2; novel phospholipides 32, 22 (120).

A fairly extensive study has been made of the novel phospholipide fractions. Traces of sphingosine nitrogen were found in certain of the fractions. However, sphingomyelin was never positively demonstrated, though its presence in trace amounts could not be completely ruled out. Novel phospholipides of haddock flesh were arbitrarily divided into three components, namely A, B and C, which occurred in the approximate ratio of 7:1.5:1.5. Lipides A and C, which were not obtained pure, had ratios of fatty acid to glycerol and phosphorus in excess of those usually found in esters of this type. They appeared to be esters of a polyglycerol phosphate in which the fatty acid: glycerol:phosphorus ratio was 4:2:1 (108, 123), corresponding to a *bis*-phosphatidic acid. However, neither of them possessed acidic properties. Fraction C, which was difficult to extract, was not identified. These three fractions also occurred in cod flesh, but in different proportions. Fractionation of the richest concentrates of inositol lipides from cod and haddock muscle indicated that the ratio of glycerol to inositol in these was approximately 2:1. They were very resistant to extraction, were present in considerably different proportions in cod and haddock muscles, and more than one type of compound was found (108, 120). Phosphatides

of serine, 2-aminoethanol and inositol have been demonstrated in the brain cephalins of Pacific pollock (125). It appears that the eggs of the same fish contain a serine phosphatide in relatively high concentration (126).

The distribution of C16, C18 and C20, saturated fatty acids, and C18, C20 and C22 unsaturated fatty acids, and the degree of unsaturation of the latter, have been reported for the following phospholipide fractions from haddock flesh: acetone-soluble lecithins, acetone-insoluble lecithins, total lecithins, and lipide fractions A, B, and C (120). The major fatty acid component of haddock depot fat, namely hexadecanoic acid, was virtually absent from all haddock lipide fractions (124). The lecithins had a remarkably high content of C20 and C22 unsaturated fatty acids, the acetone soluble lecithins having in general a greater degree of unsaturation than the acetone insoluble lecithins. The acetone-soluble lipide fraction of the eggs of a shark has been found to contain C14, C16, C18, and C20 saturated fatty acids, and C16, C18, C20, C22 and C24 mono- or polyunsaturated fatty acids. Cholesterol, chimyl and batyl alcohols were isolated from the unsaponifiable fraction (127). The egg lecithins of pollacks yielded C14, C16, and C18 saturated fatty acids, and C16, C18, C20, and C22 unsaturated fatty acids (128).

Enzymes

Seasonal variations in proteolytic enzyme activity of skipjack tuna digestive tracts have been observed (129). Crystalline pepsins have been prepared from the stomachs of salmon (130) and three species of tuna (131). These differed from hog or beef pepsins in substrate specificity, crystal structure and amino acid composition. The presence of a cathepsin has been demonstrated in the stomachs of certain fresh-water teleost fish (132), while the gastric mucosa of an elasmobranch fish has been found to possess both a cathepsin and a pepsin with pH-activity optima of 2.0 and 4.5, respectively (133).

Post-mortem proteolytic activity in fish tissues has been investigated to a limited extent, bacterial growth in whole fish muscle during prolonged holding periods being inhibited by chemical preservatives (e.g., toluene), or by prior sterilization of the tissues by ionizing radiations. With one exception (134), slow proteolysis has been observed as judged by increases in tyrosine-, ammonia- or amino-N (97, 135, 136). A recent investigation in which extracts of tissue homogenates were used for determining "cathepsin" (pH 4.0) and "neutral protease" (pH 7.6) activity, showed that the latter was absent in muscles or organs of several widely different species of fish. On the other hand the muscles, and more especially the liver and spleen, possessed much more marked cathepsin activity than did similar mammalian tissues (137).

An anserinase enzyme (pH optimum 6.3–7.5) has been demonstrated in cod muscles and partially purified (138, 139). These muscles also possess another dipeptidase (pH optimum 3–4) which hydrolyzes carnosine and

certain other dipeptides (140). A glycylglycine dipeptidase has been found in fish muscles (137). Further investigation concerning the occurrence, specificity, and properties of these peptidases is needed.

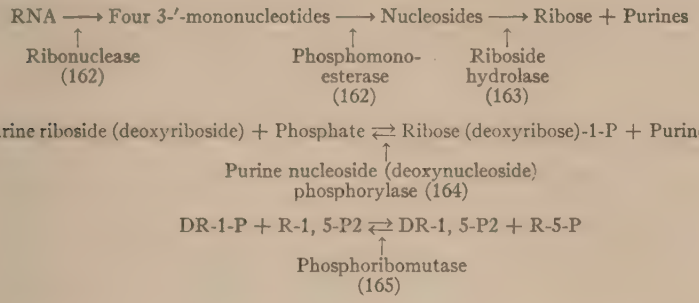
Arginase has been reported to occur in red lateral muscles of tuna (141) and in dogfish, but not in skate muscles (142). Amino acid decarboxylases have been variously reported as being probably absent (137) and present (143) in fish muscles. Catalase has been found in red lateral muscles, white muscles and livers of fish, and its seasonal distribution and some of its properties investigated (144). Carbonic anhydrase activity has been demonstrated in tissues of teleost fishes (145), and is said to play an important part in brachial excretion of bicarbonate in dogfish (146). Trimethylamine-oxide reduction, long thought to be associated solely with cells of bacteria occasioning post-mortem fish spoilage (147) has now been shown to occur in sterile fish muscles (134, 148, 149). Trimethylamineoxide is readily reduced at 20–22°C. in the presence of certain nonenzymic catalysts (150) which are almost certainly present in fish muscles, and especially in their red lateral muscles, so that the phenomenon may be nonenzymic in origin.

The glycogen content of fish muscles varies with the season, decreases markedly when fish struggle prior to capture, and the quantity present has reportedly never exceeded 850 mg. per 100 gm. (96, 151 to 154). Post-mortem glycogenolysis results in the formation of lactic acid, which is more rapid at 15°C. than at 0°C., but which, in the temperature range 0° to −10°C. is maximal at about −3°C. (153, 154). Free reducing sugar (152), recently identified as glucose, occurs in fish muscles in concentrations which range from 1 to 74 mg. per 100 gm. (155, 156). There is some confusion regarding the origin and fate of glucose in fish muscles post mortem. Thus it has been found that, in certain fish frozen in liquid nitrogen, the muscle glucose content is negligible, and that it increases markedly after a few days at 0° (156). On the other hand, it has been stated that glucose is normally present in cod muscles at time of slaughter, and decreases very slowly at 0° due to bacterial action (155). In fish liver both lactic acid and reducing sugar accumulates post mortem (157). The enzymic steps which are involved in the above degradations remain to be determined.

Fish muscles possess marked ATPase activity which, especially at 0°C., is considerably greater than that of warm-blooded animals (11, 97, 158). Two actomyosin fractions having ATPase activity with optima at pH 9 and at pH 6.5 or 7.0 have been isolated from carp muscles and certain of their properties studied (159). A decrease in ATP, an increase in orthophosphate, and formation of inosine, AMP and NH_3 were reported in muscles of frigate mackerel which had struggled prior to slaughter (160). This indicated that these muscles probably possess an ATPase, ADP deaminase and a phosphomonoesterase. No appreciable decrease in ATPase activity has been demonstrated in fish muscles stored at −18°, −76° and −190°C. for several months (161).

Many of the enzymic steps leading to the post-mortem formation of

ribose and pentose phosphate esters in Pacific lingcod muscles have been demonstrated as indicated below, and a number of the enzymes concerned partially purified.


RNA ⟶ Four 3-′-mononucleotides ⟶ Nucleosides ⟶ Ribose + Purines
Ribonuclease (162)
Phosphomono-esterase (162)
Riboside hydrolase (163)
Purine riboside (deoxyriboside) + Phosphate ⇄ Ribose (deoxyribose)-1-P + Purine
Purine nucleoside (deoxynucleoside) phosphorylase (164)
DR-1-P + R-1, 5-P2 ⇄ DR-1, 5-P2 + R-5-P
Phosphoribomutase (165)

The phosphoribomutase enzyme effected an equilibrium between R-I-P, R-5-P, DR-1-P, DR-5-P, R-1,5-P2 and DR-1,5-P2. Guanosine, adenosine (adenine) deaminase (166) and phosphodiesterase enzymes have been demonstrated in lingcod muscles (167). The comparative importance of RNA and ATP as initial substrates for the post-mortem enzymic formation of ribose in fish muscles remains to be determined, though the possible steps leading to formation of this pentose from ATP in cod muscles have been outlined (168). Free ribose accounts for much of the Maillard type of browning which may occur in heated or dried fish muscles (169, 170), and enzymically formed 1-methylhistidine may also be involved to some extent (171, 172). Alkaline phosphatase has been found in the gills (173) and kidneys (174, 175) of certain fish.

Some of the enzyme systems of fish tissues which are involved in intermediary metabolism have been investigated. The addition of pyruvic or oxalacetic acids to certain fish muscles has been found to support formation of citric acid, which occurs in concentrations of between 2.6 and 50 mg. per 100 gm. (176). A malonic acid decarboxylase has been found in muscles of carp and other fish (177). In muscles and livers of carp and minnows evidence has been accumulated which indicates that many of the enzymes concerned in the citric acid cycle, in glycolysis (e.g., glucose-6-phosphate and 6-phosphogluconate dehydrogenases), and in oxidation of fatty acids (mitochondrial enzymes) are present (178). Qo_2 values have been determined for tissue slices from adult coho salmon using different substrates in a Kreb's medium II (179), and the following decreasing order of activity found: brain, heart, liver, kidney, red lateral muscle, and ordinary skeletal muscle (180)

Nutrition

The environment in which fish live has made a study of the nutrition somewhat more complicated than is the case with most warm-blooded experimental animals. Some of the conditions which should be observed for

proper care of hatchery fish during nutrition experiments have been outlined (181). It has been stated that the protein requirement of fish is high, and the carbohydrate requirement low, in comparison with warm-blooded animals (182). High fat diets, especially those containing rancid fats, are undesirable (183). The mean composition of 69 different salmonoid fish production diets has been recorded as follows (per cent): protein, 62.4; lipide, 23.0; carbohydrate, 4.8, and ash, 10.0 (184). The exact mineral requirements of fish are difficult to establish since absorption of inorganic ions from the surrounding water occurs (183, 185, 186). The only recognized mineral deficiency in fish arises from lack of dietary iodine, but it has been assumed that fish probably require all mineral elements that are necessary to support the life of warm-blooded animals (183).

Vitamin requirements of fish have been studied by a number of investigators, but much of the earlier literature is confusing and contradictory. Young trout do not appear to require either vitamin A or D for normal growth (187, 188). The first significant work concerning the requirements of fish for vitamins of the "B complex" was carried out using a synthetic control diet, and it was found that rainbow trout required pantothenic acid, PGA, and inositol but not vitamin B12, α-tocopherol, biotin, nicotinic acid, ascorbic acid or vitamin K (189). Using this basal diet it was subsequently reported that brook trout (190) and brown trout (191) require choline, nicotinic acid, PGA, inositol, pyridoxine, and riboflavin. They also appeared to require biotin (191), but not vitamin B12 or PAB (190, 191). Recent work, in which chinook salmon fry were fed a synthetic ration which contained vitamin free casein, tryptophan, methionine, purified corn oil, minerals and vitamins, has shown that withdrawal of certain of the vitamins from the ration causes deficiency symptoms. Under the experimental conditions a definite requirement for thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, inositol, biotin, PGA and choline, but not for ascorbic acid, PAB or vitamin B12 was demonstrated (192).

The first report concerning the amino acid requirements of fish has appeared recently. Using a synthetic diet (193) it was found that chinook salmon fingerlings required nine "essential" amino acids, namely, arginine, histidine, lysine, leucine, methionine, phenylalanine, threonine, tryptophan and valine. No requirement for alanine, aspartic acid, cystine, glycine, glutamic acid, proline, serine and tyrosine was demonstrated (194). The utilization of carbohydrates orally administered to brook trout has been investigated. Absorption rates, blood sugar, liver glycogen and muscle glycogen were determined, and the danger of feeding high levels of carbohydrates to fish, which usually results in high glycogen concentrations in the livers due to the inability of fish to excrete carbohydrate, was stressed (195). It has long been known that fish absorb sugars from the surrounding water, and recent studies have dealt specifically with absorption of glucose (196), glucose and sucrose (197), and glucose, galactose and pentoses (198).

The absorption or ingestion (199 to 204), distribution (205 to 209),

and elimination (204, 208) of inorganic ions in fish has been studied extensively. Many of the investigators have used radioactive tracer techniques, and some consideration has been given to "tagging" fish in this way (210, 211). The many attempts to increase growth of young fish by inclusion of antibiotics in their diets have resulted in either no stimulation or a slight depression of growth (212).

Sterols of Marine Invertebrates

There have been two reviews on studies of the sterols of marine invertebrates prior to 1951 (213, 214), so that the present discussion has been devoted primarily to advances since that time. Particular emphasis has been placed on Japanese studies because as yet much of this work is unavailable in English. Much of the Japanese effort has been directed to a screening program involving a large number of classes of marine invertebrates, and the results are of a preliminary nature (215 to 221).

Mollusca.—Two species of chiton (Amphineura) have been shown to contain Δ^7-cholestenol (222, 223). The sterol of one fresh-water snail (Gastropoda) is reported to be β-sitosterol (224), while another is said to contain brassicasterol (7-dihydroergosterol), poriferasterol (24, α-stigmasterol), cholesterol and γ-sitosterol (225). Two other Gastropoda are reported to contain cholesterol (226).

In few fields has the use of trivial names caused as much confusion as have those given to unhomogeneous or poorly characterized marine sterols. Ostreasterol has been claimed to be identical with a sterol isolated from a sponge and named chalinasterol (213, 214, 227). Chalinasterol, in turn, was supposedly shown by ozonolysis to be 22-dehydrocampesterol (228), and on the basis of physical properties the sterol of two sea anemones was said to be identical with chalinasterol (229). The sterols of the oyster and other bivalves were shown to be complex mixtures from which a pure sterol could not be isolated by a few simple crystallizations (230, 231). Employing chromatography, 24-methylenecholesterol was shown to be a major sterol component of several bivalves and this structure was confirmed by partial synthesis (230, 231, 232). Other data support the conclusion that ostreasterol is a mixture of 24-methylenecholesterol and other di-unsaturated sterols (233). The sterol from one of the sea anemones, originally reported to be chalinasterol, has now also been shown to be 24-methylenecholesterol and apparently contains none of the sterol which gave methylisopropylacetaldehyde upon ozonolysis and on which the original structure of chalinasterol was based (234). Therefore, it has been demonstrated that chalinasterol, named for the sponge from which it originated, is not a chemical entity.

The presence of $\Delta^{5,7,22,25}$-cholestatetraenol (235) has been demonstrated in the sterols of a clam. A sterol isolated from several species of clam and named corbisterol has been shown to be 7-dehydrostigmasterol (236 to 241). However, brassicasterol, poriferasterol, γ-sitosterol and cholesterol have been indicated to be components of bivalve sterols (226, 242, 243). Since none of

the investigators was aware of the presence of 24-methylenecholesterol (230, 231) in Pelecypoda, and since most identifications have been based primarily on physical properties, it would seem undesirable to attempt to draw conclusions concerning the remaining sterols of bivalves at the present time. It is known, for example, that the sterols of at least one species of mussel and clam gave isopropylacetaldehyde and methylisopropylacetaldehyde, and no ethylisopropylacetaldehyde upon ozonolysis which indicates the presence of a Δ^{22}-sterol in the cholesterol series but no poriferasterol (244).

Echinodermata.—The comparative biochemistry of the sterols and other components of Echinodermata has received considerable attention (213, 245). It is of interest that one species of Ophiuroidea is reported to contain β-sitosterol (246) and another species to contain β-sitosterol, poriferasterol, γ-sitosterol and stigmasterol (247) rather than cholesterol (213). Thus Echinoidea are the only Echinodermata known to contain cholesterol, and this has been confirmed for another sea urchin (248). The sterols of starfish (Asteroidea) have long been known to be of the 7-unsaturated type. Delta 7-cholestenol, previously found in mammalian skin (249) has been isolated from a starfish (250, 251, 252). Hitodesterol isolated from starfish sterols has been shown to be identical with α-spinasterol (7,22-stigmastadienol) (253, 254, 255). Delta 7-stigmastenol, a recognized plant sterol (256) has also been found in a starfish (257, 258).

Crustracea.—Cholesterol has been identified as the sterol of a fresh-water lobster (Malacostraca) (259). A postulated important intermediate in cholesterol biosynthesis, 24-dehydrocholesterol, has been isolated from a barnacle (Entomastraca) and synthesized (260, 261). There appears to be some doubt whether a brine-shrimp should be considered as a primitive entomostracan or malacostracan. It is becoming apparent that evolutionary advancement correlates with increased cholesterol or a change to cholesterol. Cholesterol is the sterol of a brine-shrimp (262) and so from a comparative biochemical viewpoint the brine-shrimp should be considered as primitive on the malacostracan rather than on the entomostracan stem.

Protochordata.—Very little is known of the sterols of this class. A tunicate is reported to contain γ-sitosterol, stigmasterol, poriferasterol and 7-dehydrostigmasterol (263). The identifications are based almost entirely on physical properties.

Coelenterata.—Two cold-water sea anemones are reported to contain predominately cholesterol (264), while another sea anemone has been shown to have 24-methylenecholesterol (214). The physical properties of still another sea anemone are reported to be consistent with those of β-sitosterol (265).

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EPIDEMIC SHELLFISH POISONING IN NEW BRUNSWICK, 1957

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In 1957, there were two epidemics of paralytic shellfish poisonings in Canada—one in New Brunswick in June, the other in British Columbia in October. It has been known for many years that bivalve molluscs such as clams and mussels, in various sections of both coasts, occasionally become poisonous and are sometimes poisonous enough to produce illness and even death of consumers. Investigation has shown many differences in conditions and times of occurrence of danger in these areas, and there is no evidence to indicate that the two outbreaks this year are related.

On the Atlantic coast there are only two regions where trouble has ever arisen and only parts of these regions have been affected. One is the Gaspé shore of the St. Lawrence River estuary, from Rimouski to Douglastown, Quebec. The other is the south and north shores of the Bay of Fundy, from Annapolis Basin to near Yarmouth Harbour in Nova Scotia and from Saint John to the international border in New Brunswick (including Grand Manan and the several other islands at the mouth of Passamaquoddy Bay).

There is better understanding of the problem in the Bay of Fundy than elsewhere in Canada. It recurs in this region almost every summer but, except for short periods of peak toxicity, the poison content of shellfish is usually so low that there is little risk in eating them.

However, records show that poison content occasionally rises much higher than normal. In 1945, for example, there were 26 poisonings in the Fundy region of New Brunswick and Nova Scotia but no deaths. In the last 75 years, three deaths have been caused by shellfish poisoning.

Since 1943, Fundy shellfish stocks have been regularly checked by the Department of Fisheries and the Department of National Health and Wel-

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fare. Based on this sampling, an exacting control has been established regulating harvesting of shellfish for market. Mussel fishing has been prohibited the year round and, during the critical seasons, dangerous clam areas have been quarantined to commercial digging. Besides this, conspicuous notices have been posted, warning the general public of the risk of taking shellfish.

After the 1945 epidemic, there were no reported cases of poisoning until the week-end of June 22-23, 1957. This latest outbreak was studied to assess government control and to improve our understanding of the malady. Interest in these matters has been intensified by the British Columbia epidemic.

Extent of Epidemic

In New Brunswick, 33 people are known to have been poisoned in 1957. The offending shellfish all came from a 20-mile section of the coast—Dipper Harbour, St. John County, to Deadman Harbour, Charlotte County. One person is known to have been poisoned late Saturday night, June 22, and 24 more on Sunday, June 23. In addition, four persons were poisoned on June 30, one was poisoned on July 3 and one on July 17. A recently reported case apparently occurred in mid-June previous to the main outbreak. This case involved the bar clam (*Spisula solidissima*), all others the soft-shell clam (*Mya arenaria*).

Several of those affected were residents of shore communities, but the majority were picnickers who ate clams which they dug while at the seashore. Four were hospitalized, a dozen received medical attention in their homes and the others recovered without medical assistance. There were no deaths.

Besides the poisoning of humans, we have a record of three cats dying—two from eating soft-shell clams at Maces Bay on July 1, and one from eating bar clams at New River Beach in mid-June. Their symptoms were essentially like those described by Medcof *et al.*[1]

Investigation of Poisonings

First knowledge of the epidemic came to us on Monday morning, June 24, through a telephone call from a Saint John, N.B., newspaper reporter, enquiring about the cause of the outbreak. Checking back immediately with the District Medical Health Officer, who advised the reporter to communicate with us, we were supplied with names of eight persons who had been poisoned and with sources of shellfish eaten.

Sampling Affected Shellfish

Within four hours of receipt of this information, we gathered samples of clams from several beds involved. The other beds were sampled by officers of the Department of Fisheries on June 17 and 24 in the course of their regular checking. Thus, clam stocks chiefly concerned in the epidemic were all sampled for assay of poison content within 24 hours of the time poisonings occurred.

Compiling Case Histories

On the next day, Tuesday, June 25, and on Wednesday, June 26, with help from Drs. Trask and Hazen, we personally interviewed 16 people who had been poisoned. At that time, their experiences were still fresh in mind and they provided reports that seemed to be as full and reliable as could be hoped for. They also gave information, which we confirmed later, on 16 other persons who had eaten clams with or without harmful effects. In this way, nine more cases of poisoning during the June 22-23 weekend were discovered. Information on cases which developed after June 23 was compiled through interviews arranged as soon as possible after news of occurrence was received.

To simplify reporting, a questionnaire was devised. It called for information on source of shellfish, their preparation before consumption and other points mentioned in Tables I, II and III. A questionnaire form was completed for each person interviewed. In a few instances where interviews could not be arranged, mailed questionnaires were completed by those concerned and returned to us. All records are summarized in Tables I, II and III.

Estimating Poison Dosages

For proper study of case histories, it is important to know the amounts of poison ingested. Dosages are conventionally expressed in mouse units (m.u.). Their calculation requires information on poison content ("toxicity") and quantity of shellfish eaten.

June 23 toxicity of Lepreau Basin clams, which caused most poisonings, can be judged only by interpolation from poison content of 100 g. samples of meats collected on three other dates—508 on June 17, 17,800 on June 24 and 11,600 on June 28. The fact that all five poisonings by clams dug on June 22 were mild (Table II) indicates that maximum toxicity had not then been reached. Scores were obviously changing so rapidly that trustworthy interpolation is impossible. For this

TABLE I.—Summary of Data on 14 Cases of Serious Shellfish Poisoning Caused by New Brunswick Clams, 1957

Case No.	Sex	Age (approx.)	Symptoms												Interval between eating and onset of illness (hours)	Duration of effects (hours)	Number of shellfish eaten (approx.)	Preparation	Source	Date fished 1957	Toxicity (raw) per 100 g.	Estimated dosage (m.u.)
			Numbness of face, lips and tongue	Numbness of fingers and toes	Nausea	Vomiting	Abdominal cramps	Diarrhea	Headache	Dizziness or giddiness	Numbness of arms and legs	Prostration	Backache	Difficulty in respiration								
1	F	35	x	x	x	x	x	x	x	x	x	x	x	x	1	18d	18	steamed	Lepreau Basin	June 23	17,800	5,500
2	F	60	x	x		x				x	x	x			½	72	21	raw and steamed	Dipper Harbour	" 23	9,850	5,500
3	F	28	x	x		x				x	x	x	x	x	½	60	6	"	Lepreau Basin	" 23	17,800	7,400
4	F	30	x	x	x		x		x	x	x	x	x		½	96	40	steamed	" "	" 23	17,800	12,200
5	M	45	x	x	x		x		x	x	x	x			4	84	30	"	" "	" 23	17,800	9,100
6	F	2				x				x	x	x			<1	24	2	"	" "	" 23	17,800	600
7	M (R)	65	x	x		x				x	x	x			<½	72	20	"	Deadman Harbour	" 30	...	...
8	M	63	x	x						x	x	x		x	½	48	12	"	Lepreau Basin	" 23	17,800	3,600
9	F	50	x	x	x		x		x	x	x				2½	72	21	"	" "	" 23	17,800	6,400
10	M	65	x	x	x	x			x						½	72	24	raw	" "	" 23	17,800	51,700
11	M	60	x	x	x				x	x	x				3	36	24	raw and steamed	" "	" 23	17,800	29,500
12	M	30	x	x																		
13	M	..	x	x					x	x	x			x	½	96	5	raw	" "	" 23	17,800	10,800
14	F (R)	37	x	x	x	x			x	x	x	x			..	..	..	"	" "	" 23	17,800	...
															<1	72	..	chowder	New River Beach(1)	" (?)	...	...

(R)—Resident of clam-producing area.
(1)—Bar clams.

TABLE II. Summary of Data on 19 Cases of Mild Shellfish Poisoning Caused by New Brunswick Clams, 1957

Case No.	Sex	Age (approx.)	Symptoms: Numbness of face, lips and tongue	Numbness of fingers and toes	Nausea	Vomiting	Abdominal cramps	Diarrhea	Headache	Dizziness or giddiness	Numbness of arms and legs	Prostration	Backache	Difficulty in respiration	Interval between eating and onset of illness (hours)	Duration of effects (hours)	Number of shellfish eaten (approx.)	Preparation	Source	Date fished 1957	Toxicity (raw) per 100 g.	Estimated dosage (m.u.)
15	F	..	x	x	x						x				½	12	12	raw	Lepreau Basin	June 23	17,800	25,800
16	M	20	x	x	x					x					inst.	18	½	"	" "	" 23	17,800	2,150
17	M	..	x	x		x					x				1½	12	12	"	" "	" 23	17,800	25,800
18	F	55	x	x	x					x					1	18	22	raw and steamed	" "	" 23	17,800	25,200
19	F	20	x	x							x				9	48	35	steamed	" "	" 22	...	...
20	F	25	x	x							x				9	24	36	"	" "	" 22	...	...
21	F	31	x												2	24	24	"	" "	" 22	...	...
22	M	65	x	x							x				9	24	36	"	" "	" 22	...	...
23	M	26	x	x											11	30	36	"	" "	" 22	...	...
24	M	50	x				x	x							8	6	12	"	Dipper Harbour	" 23	9,850	1,900
25	M	60													8	6	15	"	" "	" 23	9,850	2,400
26	M	8	x												½	12	10	"	Lepreau Basin	" 23	17,800	6,300
27	M	..			x	x									..	<7	24	"	" "	" 23	17,800	7,300
28	M	..			x										3	<10	15	"	" "	" 23	17,800	4,500
29	F (R)	65	x												½	12	..	"	Deadman Harbour	" 30	...	...
30	M (R)	40	x		x				x						½	> 12	..	"	" "	" 30	...	...
31	F (R)	38	x												8	48	9	"	" "	" 30	...	...
32	M (R)	..	x	x						x	x				..	..	..	...	Foley Cove	July 17	...	...
33	F	21	x	x						x					<4	12	..	fried	Saint John	" 3	...	...

(R)—Resident of clam-producing area.

TABLE III.—Records of Consumption of Toxic New Brunswick Clams Without Harmful Effects, 1957

Case No.	*Sex*	*Age (approx.)*	*(1) Cross-ref. case No.*	*Approx. No. clams eaten*	*Preparation*	*Source*	*Date fished*	*Toxicity (raw) per 100 g.*	*Estimated dosage (m.u.)*
34	M	50	9	6	steamed	Lepreau Basin	June 23	17,800	1,800
35-37	M	7–14	9	12	"	" "	" 23	17,800	3,600
38	M	30	4	24	"	" "	" 23	17,800	7,300
39-40	M	7 and 4	4	4	"	" "	" 23	17,800	1,200
41	M	40	1	30	"	" "	" 23	17,800	9,100
42	M	55	12	32	raw and steamed	" "	" 23	17,800	13,400
43-58	M & F	adults and children	12	12	steamed	" "	" 23	17,800	3,600
59	F	60	10	6	raw	" "	" 23	17,800	12,900
60	M	31	18	24	steamed	" "	" 22	....	....
61	M(R)	13	7	10	"	Deadman Harbour	" 30	....	....
62	F(R)	15	7	9	"	" "	" 30	....	....
63	M	35	18	35	"	Lepreau Basin	" 22	....	....
64	M	10	18	..	"	" "	" 22	....	....
65	F	..	18	35	"	" "	" 22	....	....
66	M	..	18	35	"	" "	" 22	....	....
67	F	30	3	..	raw and cooked	" "	" 23	....	....
68	M	22	33	12	fried	Saint John	July 3	....	....
69	M(R)	38	14	..	chowder	New River Beach	June ?	....	....
70	F(R)	11	14	..	chowder	" "	" ?	....	....

(1)—These numbers refer to patients listed in Tables I and II who shared the same meal of clams.
(R)—Resident of clam-producing area.

reason, we have not attempted estimating poison dosages from clams taken on June 22. The highest score was recorded on June 24, just 24 hours after most poisonings occurred. Scores had fallen off by June 28 but on June 23 they must have been close to maximum—17,800 mouse units. This value has been used in calculating poison dosages which involved Lepreau Basin clams dug on June 23 (Tables I, II and III).

Scores for the lot of Dipper Harbour clams which caused the three poisonings attributable to that area were determined more precisely. These clams were dug on June 23 and taken home to Saint John. Only a portion was cooked and eaten and the others were discarded as soon as it had been discovered that they were poisonous. On June 25, most discarded clams were still alive and in good condition when recovered from a garbage can. There were enough sound ones to supply duplicate 100 g. samples of meats for extraction. These scored 10,200 and 9700 (average 9850). From what is known of effects of storage,[1] it is reasonable to assume that the score on June 23 was not less than the mean score for June 25, i.e. 9850. This value was used in calculating Dipper Harbour dosages listed in Tables I and II.

We discovered the four June 30 Deadman Harbour poisonings on July 10 and the single July 17 Foley Cove poisoning in late July. Toxicities were declining then, and both areas were under quarantine; therefore neither area was being regularly sampled. Consequently, reliable interpolation of toxicity scores is impossible and no poison dosage estimates have been attempted. The mid-June poisoning by New River bar clams did not come to our attention for some months after its occurrence.

In providing case-history information, persons interviewed estimated the number of clams eaten. To translate these into weights of clam meats ingested for poison-dosage estimates requires knowledge of meat-yield of clams available to consumers. To supply these data, a half-barrel lot of clams in the shell was obtained from Lepreau Basin in mid-July. These were studied for size-frequency composition, and meat-yield in the raw and cooked state (steamed 15 minutes). Results are listed in Table IV. Average shell length was 2½ inches (63 mm.) and meat-yield for this size of clam was 12.1 g. raw and 5.7 g. cooked. Since no information could be obtained on sizes of clams actually eaten, values for average-size clams were used to calculate weights of Lepreau Basin clam meats consumed.

TABLE IV.—Size-Composition of Half-Barrel Sample of Lepreau Basin Clams Harvested July 18, 1957, and Meat-Yields as Determined from Raw and Cooked Lots of 100 Clams of Various Sizes

Shell length (inches)	*No. of clams*	*Frequency %*	*Raw meat-yield/clam (grams)*	*Cooked meat-yield/clam (grams)*
2 - 2¼	345	29	6.3	3.8
2¼ - 2¾	645	57	12.1	5.7
2¾ - 3½	205	17	21.3	11.8

Size and meat-yield of offending Dipper Harbour clams were more satisfactorily determined from the sample recovered from garbage. These clams were more uniform in size than those in the half-barrel Lepreau Basin sample. Average shell length was 2½ inches (63 mm.) and yield of raw meat 9.9 g.

Poison dosages in mouse units, for those who ate raw clams, were calculated by multiplying total weight in grams of clam meats consumed by poison content per gram of raw meats. This latter was determined from assays by the Laboratory of Hygiene of extracts of 100 g. samples of raw meats.

Medcof *et al.*[1] showed that, on the average, 100 g. of cooked meats contains only 30% as much poison as 100 g. of raw meats. Dosages for those who ate cooked clams were calculated by multiplying the weight in grams of cooked meats consumed by 30% of the toxicity per gram of raw meats from the same source.

Poison dosages calculated as described above are entered in Tables I, II and III.

Dosages and Symptoms

Comparison of records with similar data presented by Medcof *et al.* (Tables 16 and 17)[1] reveals no essential differences. Symptoms and their order of appearance in cases of different severities are similar.

Our Table IV shows that in 1957, as in 1945, several people ingested relatively large amounts of poison (up to 13,000 mouse units) and exhibited no symptoms of illness. Tables I and II show that mild and severe cases developed from both large and small doses (see Case 6, Table I, and Case 24, Table II). Records from the 1945 epidemic show much the same picture.

Nevertheless, Medcof *et al.*[1] in interpreting their findings deduced that some people are tolerant to poison and that those who are not suffer in proportion to the amount of poison ingested. This way of thinking was clearly indicated by their listing of average dosages required to produce

mild, severe and extreme symptoms of poisoning (p. 28). From the vast differences in susceptibility, it now appears that listings of average dosages have only academic interest. To emphasize this, reference is made to Case 6 (Table I). In this instance a two-year-old child suffered serious poisoning from a dosage of only 600 m.u. Similarly, Cases 16 and 24 (Table II) involve adults who reported experiencing mild symptoms from dosages of only 1900 and 2150 m.u. respectively.

Medcof *et al.*[1] obtained some evidence that tolerance to poison may be acquired by residents of shore communities in clam-producing areas, by regularly eating mildly toxic clams. Records for 1957 merely show that there are differences in susceptibility among both residents and non-residents of such shore communities.

The most severe poisoning observed (Case 1, Table I, attended by Dr. Edgar) resulted from a 5500 mouse unit dosage. Suffocation is the immediate cause of death in many fatal cases of shellfish poisoning and, when first stricken, this woman experienced extreme difficulty in respiration. Besides this, her teeth became loose. This has never before been reported in cases of shellfish poisoning, although a loose-tooth sensation is a symptom of poisoning by certain Pacific tropical fish.[2] Dr. Edgar's patient had an unusually prolonged illness —18 days. She recovered slowly but otherwise normally until the fourth day when she suffered a complete recurrence of all her symptoms of poisoning, almost as acute as on the night of her first collapse. A relapse of this sort has never been reported. Looseness of the teeth persisted and before her recovery three actually came out. Halstead (personal communication) believes this to be the first record of loss of teeth from fish or shellfish poisoning.

The young man referred to as Case 16 (Table II) provided information of particular interest. He noted a pins-and-needles feeling in his lower lip and tongue while chewing the belly portion of a single raw clam—estimated dosage 2150 m.u. It is notable that this reaction did not discourage him from swallowing, and he later experienced further symptoms of mild poisoning.

The senior author verified this striking reaction. A red mussel (*Volsella modiolus*)—estimated poison content 1000 m.u.—was thoroughly chewed but not swallowed. Within five minutes a pins-and-needles feeling was noted around the upper and lower lips. This effect persisted for roughly half an hour but no further symptoms appeared Shantz[3] observed the same symptoms on tasting

purified poison. He adds that the purified poison appears to have no particular taste when applied to the tongue at levels sufficiently high to produce numbness.

The immediate appearance of numbness in the mouth area indicates that the poison is capable of being absorbed through the oral mucosa as well as through the stomach and intestine. This may explain why numbness of the lips, tongue and throat is mentioned in almost every recorded case of poisoning, and why this combination of symptoms appears before any others.

These rapid reactions and the severe poisoning of a two-year-old child (Case 6, Table I) from a 600 unit dosage, emphasize the extreme sensitivity of some persons to shellfish poison.

Case 14 (Table I) is also of special interest because it is our first resulting from bar clams. These are seldom eaten in the Fundy region but have long been known to be poison carriers in the New River Beach area. During the 1945 outbreak, this species scored 20,200 m.u. per 100 g. Although we made no assays, we are sure from the symptoms and their order of appearance in Case 14 that these clams were also toxic in 1957.

Comparison of 1945 and 1957 Epidemics

It is instructive to compare the 1945 epidemic[1] and the one just described (Table V). This table includes certain 1957 observations not mentioned above. For example, regularly collected samples of clams from the Nova Scotia side of the Bay of Fundy showed no toxicity; plankton samples taken in affected areas at the time of the epidemic showed unusually great numbers of the dino-

TABLE V.—Comparison of 1945 and 1957 Epidemics of Shellfish Poisonings in the Bay of Fundy Region

Feature compared	*1945 epidemic*	*1957 epidemic*
Total number of humans poisoned.	26	33
Severe poisonings...	14	14
Deaths.....	0	0
Areas involved.....	Nova Scotia and New Brunswick	New Brunswick only
Toxicity of clams chiefly involved..	18,000	17,800
Minimum raw score of clams causing illness.....	6,200	9,850
Date of outbreak...	August 16	June 22 - 23
Water temperatures	Sudden rise preceded outbreak	Sudden rise preceded outbreak
Frequency of *Gonyaulax tamarensis*.....	Abundant in plankton	Abundant in plankton

flagellate *Gonyaulax tamarensis* Lebour, which is believed to be the causative agent; a sudden rise in water temperatures occurred just previous to the outbreak and, finally, clams from many areas in the Fundy region not referred to here, were regularly sampled before, during and after the outbreak as part of the federal government's management scheme.

Assay results for these samplings are not exhibited here; but, in most New Brunswick areas concerned, scores reached approximately the same relative levels during both epidemics. Seemingly, worst areas are always worst and best areas best. In this respect, east coast conditions seem to differ remarkably from those in British Columbia and on the west coast as a whole. There, no such clear patterns have been reported.

Table V shows that the two epidemics in the Fundy region were roughly equal in severity, judging from the numbers of serious poisonings. The focus of trouble in both was Lepreau Basin, and toxicity scores of offending clams from this area were the same to within 200 units. In both epidemics, poisonings resulted from consumption of highly toxic clams; in 1945 the minimum score of offending clams was 6200 m.u. per 100 g. of raw meat, and in 1957, 9850. The same plankton organism was apparently responsible for toxicity of shellfish. Unusually warm weather which induced a sudden rise in water temperatures preceded both outbreaks. In 1945, shellfish on both Nova Scotia and New Brunswick sides of the Fundy were affected, but in 1957 only those on the New Brunswick side were toxic. (This is an unexplained peculiarity.) The 1945 epidemic occurred in mid-August, whereas that of 1957 was in late June.

Similarities of the two epidemics are striking, differences less remarkable. In many respects this is fortunate. It means that, compared with Pacific coast investigators, we are dealing with a less variable problem and one that may be managed with less effort.

Management Problems

Critique of 400-Unit Quarantine

From what we know, it is theoretically possible to suffer poisoning from eating shellfish with low toxicity scores, but we have never observed this. The Canadian Department of National Health and Welfare and the United States Department of Health, Welfare and Education have agreed that

shellfish with scores up to 400 m.u. per 100 g. are safe. Accordingly, on the basis of regular tests, our areas are closed for commercial harvesting and marketing of raw shellfish as soon as this level is approached. Similarly, quarantined areas are reopened as soon as controlling agencies have satisfied themselves that scores have fallen below the 400-unit level and are likely to remain there. This investigation has discovered nothing that would discourage continuance of this practice. On the other hand, we have no substantial grounds for liberalizing the use of toxic shellfish by increasing the maximum 400-unit limit.

Prediction

In 1957, the interval between first rises of toxicity in dangerous areas and further rises to hazardous levels was short; but there was time to permit display of warning notices for the instruction of those who cared to heed signs. However, it must be admitted that the interval was dangerously short and some system of earlier warning is needed.

Needler[4] found that *Gonyaulax tamarensis,* the plankton organism responsible for toxicity, appeared only after the surface water temperatures rose to 10° C. and disappeared quickly when shellfish toxicity scores began to decline. From the last 14 years' records, Bond[5] has demonstrated an inverse correlation between mean annual toxicity scores and winter water temperatures. Besides this, hydrographers[6] have demonstrated a downward trend in water temperatures in recent years, and predict that, during the next 20 to 25 years, water temperatures will continue to be low. In other words, poison problems are likely to be more acute, and the need for earlier predictions more urgent than in recent years.

Research Needed

Present bioassay methods for measuring toxicity of shellfish involve several days' delay between collection and extraction of shellfish samples at the coast, their shipment and, finally, their assay at the Laboratory of Hygiene in Ottawa. Chemical methods of assay are being devised,[7, 8] and if they can be simplified for quick determinations of toxicity at the coast, this would provide earlier warning.

Regardless of relative merits, both assay methods have an essential weakness. The causative organism

must have appeared in the water and have been fed on by shellfish before either can give warning of serious rises in toxicity. Experience has shown that populations of the causative organism are subject to violent fluctuations. In other words, neither method can be counted on for sufficiently early warning. Perhaps Needler[4] and Bond[5] have pointed the way to better methods. Their studies indicate that year-to-year differences in water temperatures determine abundance of the causative organism and, hence, toxicity of shellfish. A good understanding of this relationship might permit a true long-range method of predicting danger. It cannot be developed, however, without intimate knowledge of the biology of the causative organism. The need for better prediction would justify research along these lines.

Public Education Needed

For 15 years now, the Department of Fisheries and the Department of National Health and Welfare have co-operated in regularly sampling shellfish stocks in the Fundy area. Every year, as soon as the first rise in toxicity is detected, conspicuous warning signs are posted in all areas which are known to become dangerous. This was done as usual in 1957. Lepreau Basin, for instance, was posted on Saturday morning, June 22; but 23 persons were poisoned by clams dug there in the next 48 hours. In interviews on the following Tuesday, several persons admitted having seen and read the warning signs. Some more convincing form of public information is required.

After June 23, newspaper coverage was excellent and we credit it with discouraging other picnickers from eating clams. Nevertheless, on June 30 and on July 17, some shore residents ate clams and were poisoned (Cases 7, Table I; 29-31, Table II). Signs and newspaper warnings were not enough.

Public education is the most critical of all management problems. It must be faced.

Summary

In June and July, 1957, soft-shell clams (*Mya arenaria*) and bar clams (*Spisula solidissima*) from a 20-mile reach of the Bay of Fundy shore in New Brunswick caused 33 poisonings, mostly among picnickers. Fourteen were severe, the others mild. Four were hospitalized but there were no deaths. Compilation of case histories and assays of poison content of shellfish (highest—17,800 m.u. per 100 g. raw meat) permitted calculations of poison dosages. Some people were tolerant to massive doses and others not. The most sensitive reacted to small doses. Loosening of

teeth, incipient respiratory failure and a recurrence of all symptoms after partial recovery were observed in the most severe case. There is evidence that the poison may be absorbed through the oral mucosa. This may explain the orderly appearance of the various symptoms of poisoning. An epidemic similar in character occurred in 1945.

The Department of National Health and Welfare and the Department of Fisheries regularly determine the danger periods and have good control of commercial harvesting of shellfish. Warning notices are posted but these have not prevented poisonings.

There is need both for better public education and for longer-range prediction of danger periods.

We are grateful for much assistance. The Department of National Health and Welfare, particularly the Laboratory of Hygiene, assayed extracts of all shellfish samples taken and kept us informed of results. Officers of several services of the Department of Fisheries helped in many ways. Dr. F. C. Hazen, Medical Health Officer for St. John and Charlotte Counties, N.B., kept us constantly informed of developments as they came to his attention. Dr. C. R. Trask, Director, General Hospital, Saint John, N.B., supplied information and arranged interviews with shellfish poison patients who were treated in his institution. Dr. A. B. Edgar of Lancaster, N.B., supplied case history details on the most severe poisoning observed. Mr. Ralph Costello, Saint John, N.B., Managing Editor of *The Telegraph Journal* and *The Evening Times-Globe*, and his staff of reporters, kept in close touch with us and Dr. Hazen. They informed the whole district of the dangers of poisoning and, without doubt, prevented much suffering.

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DOLPHINS IN NEWFOUNDLAND WATERS

by

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DOLPHINS IN NEWFOUNDLAND WATERS

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SERGEANT and Fisher (1957) recorded the presence of common dolphins *Delphinus delphis* L. on the offshore banks of Nova Scotia in late summer and autumn 1954, the species apparently being common at this time of year in the region of 'slope' water which lies between the Gulf Stream and the colder waters of the Nova Scotian coast. Sea surface temperatures recorded at the localities of sighting were between 13 and 17°C.

The records described below extend the distribution of *Delphinus* northward in the northwest Atlantic to the region of Newfoundland.

(a) A male common dolphin was shot in Dildo Arm, Trinity Bay, Newfoundland (47° 32′ N., 53° 33′ W.), on July 22, 1957, by the crew of the whaling vessel *Arctic Venture* owned by the Department of Fisheries of the Province of Newfoundland. The ship was at the time engaged in driving to shore a herd of some 155 pilot whales *Globicephala melaena* (Traill), and the dolphin though evidently alone of its kind was associating with the pilot whales. The pilot whales were the first to be seen and captured in 1957 in Trinity Bay, the site of an extensive fishery for this species. For a description of the fishery for pilot whales in Newfoundland and their migrations, reference is made to the paper mentioned above.

The dolphin was examined by the writer a few hours after capture (Figure 1), and circumstances permitted rather full examination of external and internal characteristics. Measurements, given in centimeters, were as follows; total standard length 190 (6 ft 3 in.); projection of lower jaw beyond tip of snout 1.3; tip of snout to center of eye 34, to blowhole 34, to angle of gape 25, to tip of flipper 79; flipper length, axilla to tip 23; maximum flipper width 11; length at base of dorsal fin from anterior end of ridge to immediately below apex 28; vertical height of dorsal fin 22; span of flukes 47; notch of flukes to anus 53; anus to penis 18; penis to umbilicus 36. The weight of the freshly killed animal was 80 kg (175 lb). The number of teeth visible in the intact animal, on the right side, was 41 in the upper jaw and 42 in the lower; in the boiled-out jaws, the left side showed 42/44 and the right 43/44. The first chamber of the stomach contained remains (beaks and pens) of at least twenty small squid *Illex illecebrosus* Lesueur, which are abundant in this region in summer and form the principal food of the pilot whales. The testes weighed each some 37.5 grams, and histological examination of testis and epididymis showed no spermatogenic activity, though sexual maturity had evidently been attained. According to Kleinenberg (1956) male *Delphinus* attains sexual maturity at a mean length of 170 cm in the Black Sea.

Externally, the sides of the body showed rows of parallel scratches, these being fine and closely set, and so judged to have been made by other dolphins and not by the pilot whales. There was a single external parasite, a specimen about 15 mm long of the aberrant sessile barnacle *Xenobalanus globicipitis*

FIGURE 1. Head of *Delphinus delphis*, male, length 190 cm, captured in Dildo Arm, Trinity Bay, Newfoundland, on July 22, 1957.

FIGURE 2. Common dolphins breaching in the bow wave of the ship, Flemish Cap, 47°00′N., 44°32′W., August 1, 1957.

Steenstrup, attached to the dorsal surface of the right tail fluke. The skull having been broken by heavy bullets could not be saved; however the jaws are now in the collection of the Arctic Unit of the Fisheries Research Board of Canada, in Montreal.

In observations of Cetacea made in Trinity Bay for varying periods each year from 1952 through 1956, the writer had obtained no previous record of *Delphinus*, although the dolphins *Lagenorhynchus acutus* and *L. albirostris* were both encountered (Sergeant and Fisher, as above). The animal was strange also to the whaling captain and mate, who have a lifetime's experience of hunting in the bay and some ten years of whaling there. *Delphinus* is known to be an inhabitant of tropical and warm temperate seas, a social species and a fish-eater. The circumstances of the present record—the solitary young male with atypical food—combine to suggest that the animal was a wanderer outside the normal range of the species. The sea surface temperature close to the place of capture on the same day was 11.3°C. The writer is indebted to Capt. Clarence George and to Arctic Fishery Products Ltd., South Dildo, Newfoundland, for allowing examination and collection of specimens from this animal.

(b) A school of *Delphinus* was identified by the writer at approximately 47° N., 44° 32′ W. (over the east side of the bank known as Flemish Cap, east of the Grand Bank) on August 1, 1957. He was then taking part in a hydrographic cruise of the M.V. *Investigator II* operated by the Biological Station of the Fisheries Research Board at St. John's, Newfoundland. The school was estimated to contain 30 to 40 individuals, which approached the ship for about 20 minutes, alternately swimming alongside, 'riding the bow wave' (Figure 2) and racing ahead, with the ship traveling at about 9 knots. Having examined the dead specimen only 9 days previously, the writer found sight identification easy, recognition characters in the water including: the black stripe from eye to beak, the buff-gray patch on the anterior flanks, the two gray streaks on the posterior flanks, and the dark brown color of the back. The upper side of the flipper in all animals appeared white, which was surprising in view of the fact that the flipper of the specimen, agreeing with the customary description, had been black. However, it was noted that the top of the beak and part of the caudal peduncle also appeared white, the extent of the white varying with time in any one individual. It was therefore deduced that the white color was the result of cavitation of water round these body areas. These observations show that the apparent color pattern of dolphins seen in the water may not always be the true pattern.

Temperature studies carried out on this cruise showed that the area of the sighting lay in water of intermediate temperature between the cold Labrador Current covering most of the Grand Bank and the warm North Atlantic Drift to the eastward. The surface temperature at the station nearest to the position of sighting, at 47°00′ N., 45°00′ W., was 11.38°C. Although temperatures close to the coast in the vicinity of St. John's were similar (11.53°C at 47° 32′50″N., 52° 35′10″W.) on July 29, surface temperatures over the main mass of the intervening Labrador Current were as low as 9°C.

(c) The species was not met with elsewhere on this cruise, which extended northward from the 47°N. parallel as far as the mid-Labrador coast. However, observations on Cetacea were made by the officers and scientific personnel of the *Investigator II* on a subsequent cruise which extended over the southern part of the Grand Bank and over St. Pierre Bank. A school of eight to ten dolphins was recorded at 42° 50′ N., 50° 05′ W. (over the Tail of the Grand Bank) on August 23, 1957. A photograph taken by Mr. C. A. Rose of one of these animals shows the unmistakeable convergence of the dark and light markings on the side of a common dolphin. The surface temperature close to this locality at 42° 56′30″N., 50° 16′45″W. was 11.10°C.

I am indebted to Dr. W. Templeman, Director of the Biological Station of the Fisheries Research Board at St John's, Newfoundland, for the opportunity to travel on the *Investigator II* and for permission to use these temperature data; to Mr. E. L. Rowe of the same Station for an enlargement of Mr. Rose's photograph, and to Dr. D. A. Parry of Cambridge University, England for pointing out the difference between turbulence and cavitation.

Sergeant and Fisher (as above) recorded billfish *Scomberesox saurus* (Walbaum) in the stomach of one of two common dolphins captured off Nova Scotia. This fish extends north to Newfoundland in numbers at the height of summer warming of the surface waters.

SUMMARY

Delphinus delphis is recorded from the southern edge of the Grand Bank, and from Flemish Cap, off Newfoundland, in late July and August. A single specimen is recorded from the coast of eastern Newfoundland in late July. All three records come from water of surface temperature between 11 and 12°C. These records extend the known distribution of the species northward in the northwest Atlantic in summer.

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

I. PHYSICAL MEASUREMENTS, PLASMA CHOLESTEROL, AND ELECTROLYTE LEVELS[1]

D. R. Idler and H. Tsuyuki

Abstract

Physical measurements have been made on all major organs and tissues of a pure race of migrating adult *Oncorhynchus nerka*. The data show that the population was sufficiently uniform in size that average weights and measurements performed on fish taken at three locations on the migration route quite accurately represented a standard fish of both sexes. The results will enable chemical analyses to be interpreted as changes in absolute amounts of a given tissue component rather than only on a percentage basis. The merits of this approach to an evaluation of changes in migrating salmon is demonstrated by the magnitude of the weight changes in many tissues during the 715-mile migration. A standard male lost only 11% of its body flesh whereas a standard female lost 30%. The alimentary tract decreased to one sixth of the initial weight. The milt increased by 51% while the roe increased by 272%. Thus, it is apparent that percentage composition data cannot show quantitative changes in a fish as it moves up the river. The results are discussed in relation to the findings of others on Salmonidae.

Plasma cholesterol, magnesium, calcium, sulphate, and urea levels were measured on pooled blood samples from the 216 fish.

Introduction

Many of the physiological and biochemical changes occurring during the spawning migration of Salmonidae have been investigated. Interest has been primarily stimulated by the fact that the fish do not feed for many weeks or in some cases months (1, 2, 3, 4, 5). This is also a time of great activity. Tissue energy reserves must be mobilized both for the enlargement of the gonads and for the trip to the spawning grounds, which are often many hundreds of miles from the sea (1, 2, 3, 4).

The expenditure of fat and protein reserves by the Atlantic salmon *Salmo salar* (2, 3) and the Pacific coast *Oncorhynchus tschawytscha* (spring salmon) has been investigated (1). None of these studies appears to provide data which would permit energy expenditures to be calculated either on a time or distance of travel basis. The reasons for this differ in each investigation and include: (1) non-continuous sampling during the spawning migration; (2) too few samples; (3) too few chemical analyses; (4) difficulties in converting

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data to a standard fish owing to large variations in the dimensions of individuals. These limitations were overcome to a large degree by the studies of Pentegov *et al.* on migrating *Oncorhynchus keta* (chum salmon) on the Amur River (4). This comprehensive study showed for the first time the energy expenditures of male and female fish per unit distance over the entire freshwater migration of approximately 700 miles. The relatively uniform size and stage of maturity of the population made it possible, within reasonable limits, to accomplish the same results as would be obtained if one could perform a complete proximate analysis on a fish, then return it to the water, and recapture it further upstream. Even this magnificent effort was hampered to some degree by the variations in individual fish which apparently were sometimes not adequately compensated for by the number of samples available. Variations in flesh depot fat in limited flesh segments have been investigated for sockeye salmon (*Oncorhynchus nerka*) on the Columbia River (6). A report of limited scope is available on fat and protein analyses for sockeye on the Harrison River system (7).

In 1956 and again this year, members of the staff of this Station collaborated with the International Pacific Salmon Fisheries Commission biologists on a program designed to study the energy expenditures of Fraser River sockeye migrants to Stuart and Chilco lakes.* The Stuart Lake run has now been investigated for 2 consecutive years. The ability of the biologists to select a pure race of fish of nearly uniform length and weight suggested an excellent opportunity to investigate the more fundamental biochemical changes in individual organs and tissues in a manner analogous to the determination of total energy reserves in the entire fish.

The distribution of flesh nitrogen compounds (8, 9), plasma, and flesh cholesterol, and certain plasma electrolyte levels has been completed. This investigation was carried out on 216 fish representing both sexes of sockeye in three different phases of migration. Proximate analyses, qualitative and quantitative distribution of phosphorus compounds, creatine, inositol, and nucleic acid levels are under investigation by various scientists at this Station. These studies will not be completed for some time.

This initial report provides necessary background information on the fish used in the phases of the project completed to date. It demonstrates also the degree of success achieved in selecting fish of uniform length and weight. There appears to be no available information in the literature for salmon concerning the electrolytes determined in the present study. Blood cholesterol has been measured on an unspecified species of sexually mature salmon taken in a river in Japan (10).

Methods

All fish were caught in reef nets to minimize struggle; they were anaesthetized in ice water before they were bled by cutting off the tail to sever the caudal artery. Heparin was added to the blood, which was centrifuged immediately and the plasma transported to the laboratory in dry ice. Individual

*It is anticipated that the results of the study will be published soon.

fish were placed in polyethylene bags and frozen in brine (−19° C.) at Lummi Island, and on dry ice at the more distant points. The fish were kept at −30° C. until they could be processed within a few days. They were thawed overnight at 0° C. by which time it was just possible to slit the belly wall and remove the frozen viscera. All tissues were kept in polyethylene bags on dry ice until the required number of organs to complete a group was obtained. Four groups of male and four groups of female fish were obtained from each location. The sizes of the groups are shown in Table I.

TABLE I

SAMPLING OF SOCKEYE SALMON

Sex	Group	Number of fish per group		
		Lummi	Lillooet	Forfar
♂	1	7	10	10
♂	2	6	10	5
♂	3	5	10	6
♂	4	5	10	4
♀	1	8	10	14
♀	2	7	10	14
♀	3	9	10	13
♀	4	9	10	14

The internal organs of each group of fish were separated, then pooled and weighed while still frozen. Each eviscerated fish was then weighed both in water and air, as was the tail. Specific gravities were calculated from these data. Each eviscerated fish was then filleted and the entire body flesh portion kept separate from the bone, skin, head, and tail.

Plasma cholesterol was determined by the procedure of Sperry and Webb (11) and sulphate sulphur was determined as described by Cope (12). Well-established procedures were employed to determine calcium as the oxalate, magnesium as magnesium ammonium phosphate, and urea with urease (13).

Results and Discussion

The physical changes in the fish from Lummi Island, where they were in water of low salt content, to Lillooet on the Fraser River and to the spawning grounds at Forfar Creek are shown in Table II. The distances are given in Table IV. Although each fish was measured and its weight recorded the large number of samples necessitates the reporting of averages only for each group. For this reason most of the data have been statistically analyzed. In the discussion "very significant" refers to probability as obtained by the "t" test of .001 or less. When p is between .05 and .001 it is described as significant. When p is greater than .05 no significance is ascribed to the data. All data are recorded as mean ± standard error. Since each group of fish was not exactly the same size, slightly different values were obtained when mean values were calculated on individual fish rather than on group averages. This was particularly true for males at Lummi Island and Forfar Creek. Because of

the complexity of the calculations individual fish were analyzed statistically only for total weight, body length, flesh weight, and weight of the eviscerated fish at Lummi Island. This was done to make the corrections to a standard fish as accurate as possible.

Changes in the Tissues and Organs

A detailed discussion of these changes will be given when proximate analyses are completed. For the present certain observations appear to be pertinent.

It is a well-known fact that the snout length of males increases significantly during migration whereas that of females increases but to a lesser extent. The present study confirms this fact and demonstrates the quantitative aspects for sockeye salmon on a very uniform size population. The male snout increased by 52% and the female by 25% during the migration (Table II). The body length would be expected to remain constant for both sexes with possibly a very small increase during migration owing to the portion of the head which was included in this measurement. The female groups (Table II) show that this constancy has been realized. The average body length of the 23 male fish taken at Lummi Island was somewhat short (49.9 cm.) but the difference was not statistically significant. This situation was anticipated and an additional 17, 35, and 18 male fish were measured at the three consecutive points. When these data were included males averaged 50.2, 50.2, and 50.3 cm. at each consecutive point. All weight data for males at Lummi Island were corrected to body length 50.2 from 49.9 cm. on the previously established basis that the body weight is proportional to the length cubed when the correction is small as it is in this instance (3). There was a significant weight loss in both males and females between Lummi Island and Lillooet. In spite of the extensive depletion of fat and protein stores the males regained their original body weight while the females lost no more on the run to the spawning grounds. Tissue water is the prime factor in explaining this observation but this will be discussed when proximate analyses are completed. The losses in fat and protein with an accompanying percentage increase in body water is well established for migrating salmon (1, 3) and results in the very significant changes in specific gravity of both sexes (Table II). A thickening of the skin during spawning migration has been observed in spring salmon (1, 5). The increase in snout length has already been mentioned. Table III shows that the tail also increased in weight. Since the fish do not feed once they begin the river migration it is not surprising that the viscera of both sexes decreased very significantly in weight. Table III shows that this loss is primarily a reflection of a nearly complete wasting of the alimentary tract. When considering the energy expended during the migration this factor should be taken into consideration. The heart and kidney of both sexes underwent relatively little change in weight. The spleen of both sexes first decreased then increased in weight. The sexes started out very nearly equal in weight when the difference in body length is taken into consideration. However, a comparison of the sexes shows that the females had significantly larger livers than the males. The males had significantly larger alimentary tracts and kidneys.

TABLE II

LENGTH AND WEIGHT CHANGES OF SOCKEYE SALMON DURING SPAWNING MIGRATION

Site	Sex	Groups	Total fish, g.	Total length, cm.	Standard length, A	Snout length, B	Body length, A–B	Evis. fish, g.	Head, skin bones, tail, g.	Flesh, g.	Sp. grounds evis. fish
Lummi Is.	♂	4	2565±42	59.3	53.6	3.4±.11	50.2±.29	2322±40	881±33	1467±21	1.033±.000
Lillooet	♂	4	2371±26	59.3	54.1	3.9±.15	50.2±.00	2185±25	871±10	1302±51	1.024±.000
Forfar Cr.	♂	4	2583±51	61.1	55.5	5.2± 16	50.3±.28	2382±47	1057±10	1304±40	1.012±.000
Lummi Is.	♀	4	2376±26	57.4	52.0	2.8±.04	49.2±.29	2151±19	785±15	1335±13	1.037±.000
Lillooet	♀	4	2156± 4	57.6	52.4	3.1±.05	49.3±.40	1908±41	765±20	1135±30	1.024±.000
Forfar Cr.	♀	4	2168±10	58.2	52.8	3.5±.04	49.3±.26	1775±10	834±12	934±10	1.015±.000

TABLE III

WEIGHT AND LENGTH CHANGES IN THE ORGANS OF SOCKEYE SALMON DURING SPAWNING MIGRATION

Site	Sex	Groups	Heart, g.	Liver, g.	Spleen, g.	Kidney, g.	Alimentary tract, g.	Viscera, g.	Gonads, g.	Tail, g.
Lummi Is.	♂	4	6.10±.43	31.6±0.8	2.90±.26	26.0±0.8	90.5±3.3	157±5.0	56.5±2.3	64. ±2.3
Lillooet	♂	4	5.88±.26	25.4±0.6	1.98±.05	23.3±0.7	30.1±0.4	86.7±1.7	82.0±2.8	58. ±0.5
Forfar Cr.	♂	4	5.80±.28	37.6±1.0	3.60±.23	28.1±1.0	16.4±1.0	91.5±2.4	85.4±2.4	82. ±3.1
Lummi Is.	♀	4	5.05±.13	40.2±0.5	2.04±.09	21.6±0.7	78.3±1.6	147±3.0	80.1±0.8	59. ±0.6
Lillooet	♀	4	4.95±.21	44.6±0.9	1.50±.08	19.9±0.2	26.2±0.8	97.2±3.6	138±3.7	52. ±1.5
Forfar Cr.	♀	4	4.58±.06	40.5±1.6	2.17±.18	19.7±0.2	12.9±2.7	79.9±2.1	298±2.3	77. ±0.9

Gonads

The energy which a starving fish must expend to produce milt or roe has been emphasized for *Salmo salar* in the rivers of Scotland (3). In May and June the ovaries represented only 1.2% of the total weight of the fish, whereas in September and October on the upper river the ovaries were 23.2% of the fish. The testes increased from 0.15% to 3.32% of the weight of the fish. Greene's data for Columbia River *O. tschawytscha* shows that the ovaries represented 5% (two fish) of the weight of the fish at the mouth of the river and 20% (four fish) on the spawning grounds but the number of fish was limited and the fish were quite variable in size. Miescher-Rüsch emphasized that abnormal proportions were exhibited between the sexual glands and the weight of the body for extreme sizes of Atlantic salmon (2). He concluded that the large majority of Rhine salmon did not develop significant milt or roe while in the sea. This is not a general phenomenon, however, as Paton has shown that both male and female Atlantic salmon entering rivers in Scotland showed large increases in milt and roe from May through October. For the ovaries this variation was from 1.2% in May and June to 12.4% in September and October. In the same time period the milt of incoming migrants increased from 0.15% to 2.71% (3).

In comparing the data for *O. nerka* with that outlined for other Salmonidae certain points are worthy of mention. The Atlantic salmon, *S. salar*, is generally a much larger fish. Thus, the fish in the present study averaged about 2.5 kg., whereas the Rhine salmon examined at Basel averaged 8 kg. The *O. tschawytscha* on which Greene carried out his studies varied from about 7.5 to 16 kg. *O. keta* investigated by Pentegov averaged around 4 kg. The Atlantic salmon frequently return to the sea after spawning whereas *Oncorhynchus* do not. The Rhine salmon spend many months in the spawning area waiting for the gonads to ripen, whereas in the present study only a few days separated arrival and spawning. However, both genera cease to take food by the time the fish reach fresh water (and probably before) and then begin an extended period of starvation at a time when the energy demands both for swimming and sexual maturation are extensive.

The study by Pentegov *et al.* on migrating chum salmon (*O. keta*) on the Amur River most closely parallels the present study (4). The total distance of the migration, somewhat over 700 miles, was very comparable (Table IV). The water temperatures were similar. The distance to an intermediate point on the migration was nearly identical. Chum salmon migrate somewhat slower than sockeye salmon. The gonads of both the male sockeye and chum salmon did not increase in weight after the first 250 miles of river migration (Tables III and IV). The gonads of both male and female chum salmon on the Amur River were heavier than those of sockeye salmon in relation to body weight on entering the river. The milt of both species appeared to decrease in relation to body weight in the later stages of migration (Table IV). For sockeye this change was not significant.

The energy expenditure to produce roe by both female sockeye and chum salmon was considerably greater than the expenditure by males to produce milt. Of particular importance was the relatively small weight of milt which must be produced by males during the starvation period in the river in comparison to the roe produced by the female (Table III).

TABLE IV

COMPARISON OF OVARIES FOR STUART LAKE SOCKEYE AND AMUR RIVER CHUM SALMON

Location	Days	Miles	H_2O temp., °F.	Milt, %*	Roe, %*
Fraser River					
Lummi Is.	0	0	62 (est.)	2.20	3.37
Lillooet	10	250	63	3.46	6.40
Sp. grounds	27	715	48	3.31	13.7
Amur River					
Langre Is.	0	0	63	4.11	7.02
Sophiskoya	12	253	62	4.21	10.1
Sp. grounds	33	740	48	3.08	17.4

*Expressed as percentage of the live weight of the standard fish at each station.

Plasma Cholesterol and Electrolytes

The levels of free and esterified cholesterol found in male and female plasma are given in Table V. The "normal" total cholesterol levels at the beginning of the upriver migration were very high (570 mg.%) compared to warm-blooded animals (150-190 mg.%) (13). However, average levels of 662 mg. % have been reported for carp blood (14). The decrease in total plasma cholesterol from Lummi Island to the spawning grounds (715 miles) was far greater in female (65%) than in male fish (31%). Females also lost more free cholesterol (64%) than did males (53%). Over the relatively short distance between Lummi Island and Lillooet (250 miles) the females showed no decrease in free cholesterol and a 13% decrease in total cholesterol. The males over the same distance lost 23% total cholesterol and 35% free cholesterol. It can thus be concluded that the females began to lose plasma cholesterol later in the migration than did the males but the losses in the later stages were much greater in the female. The ratio of free to total cholesterol steadily decreased as the males moved up the river. The ratio was 0.46 at Lummi Island, 0.39 at Lillooet, and 0.34 at the spawning grounds. The females show less variation in the ratio of free to total cholesterol. The ratio was 0.38 at Lummi Island and Fortar Creek and 0.44 at Lillooet. On arrival of the fish at the spawning grounds, which for this race occurs only a few days before spawning, both the total and free cholesterol levels were significantly higher in the male. However, for an unspecified species of sexually mature salmon (probably *O. keta*) in a Japanese river, similar levels of both free (75-80 mg.%) and total cholesterol (150 mg.%) have been reported for both sexes (10). The present

TABLE V

PLASMA CHOLESTEROL AND ELECTROLYTE LEVELS IN MIGRATING SOCKEYE SALMON

Components, mg.%	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Free cholesterol	♂	261±18(23)*	170±17(40)* .02†	134±15(25)* .1†
	♀	215± 9(33)*	220±24(40)* .8†	77± 6(55)* .001†
Total cholesterol	♂	570±19	436±38 .02	394±25 .4
	♀	572±19	499±24 .05	202±20 .01
Calcium	♂	21.6±0.6	19.9±1.3 .2	12.5±0.8 .001
	♀	27.3±0.4	23.8±1.1 .02	15.5±0.6 .001
Magnesium	♂	1.58±0.16	3.71±0.25 .001	2.15±0.11 .01
	♀	0.86±0.06	2.88±0.18 .001	2.18±0.12 .01
Urea N	♂	6.0±0.3	4.3±0.7 .05	4.9±0.2 .3
	♀	6.2±0.6	3.7±0.5 .02	4.2±0.4 .4
Inorganic sulphate as S	♂	12.5±0.2	5.5±0.3 .001	9.6±0.3 .001
	♀	13.0±0.7	3.9±0.3 .001	9.8±0.2 .001

*Total number of fish divided into four groups.
†Significance of difference from value immediately to left.

study has shown that the plasma cholesterol levels were extremely high in sexually immature sockeye salmon and decreased with increasing sexual maturity and/or decreasing energy reserves.

The carbohydrate and glycogen content of salmon liver and flesh is known to be small (15). However, it was considered of interest to determine levels of plasma magnesium. The results (Table V) show that they were generally within the range of 1–3 mg.% reported for human serum (13). Carp serum is reported to contain 3.32 mg.% (14). The very significant increase during the migration may reflect an increased carbohydrate metabolism during this very active phase of the salmon's life, but this is purely conjecture. The recent isolation of a new sulphate-containing nucleotide from salmon liver in this laboratory prompted the determination of plasma sulphate (16). The levels (Table V) at all stages of migration were very significantly higher than those reported for human serum (0.9–1.1 mg.%) (13) or carp serum (0.94 mg.%) (14). The difference between the initial and final levels can probably be accounted for by blood dilution, which is reported to be about 12% for *O. tschawytscha* (1). The significant drop during the early phase of migration

and the subsequent rise in the later phase is analogous to the change reported in human pregnancy (17). Urea nitrogen levels, 6 mg.% for males and 6.2 mg.% for females, were lower than those normally reported for humans (10–15 mg.%) (13) but somewhat higher than for fresh-water carp (3.5 mg.%) and trout (2.6 mg.%) (14). The changes during spawning migration can probably be largely accounted for by dilution of the blood. The calcium levels, 21.6 and 27.3 mg.%, respectively, for males and females, were much higher than the 9–11.5 mg.% reported for man (13) or the 11.5 mg.% reported for carp serum (14). The calcium levels like the cholesterol levels decreased in the final stage of sexual maturity to the same magnitude as is found in humans. Parathyroid-like function has been attributed to the ultimobranchial gland of fish (18, 19, 20). However, too little is known of the functions of this gland at the present time to justify more than a suggestion that decreased activity of the gland may be associated with the continually decreasing blood calcium levels. By analogy with mammalian serum the decreased calcium levels should be accompanied by a decrease in serum protein (21, 22). This will be investigated.

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NUTRITION AND METABOLISM OF MARINE BACTERIA

VII. GROWTH RESPONSE OF A MARINE FLAVOBACTERIUM TO SURFACE ACTIVE AGENTS AND NUCLEOTIDES

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In the course of a study of the nutritional requirements of a number of marine bacteria, one was found which failed to grow unless a supplement of yeast extract or other natural materials was added to the medium (MacLeod *et al.*, 1954). A chemically defined medium suitable for the growth of this organism has now been obtained. In a medium containing 18 amino acids and 2 vitamins, the growth-promoting effect of yeast extract for the organism could be largely but not completely replaced by a mixture of a surface active agent and 3 nucleotides. The development of the medium and the nature of the response of the organism to the various growth-promoting agents is presented below.

METHODS

Culture. The organism, designated B4 in the previous communication (MacLeod *et al.*, 1954) was isolated from sea water and has been tentatively identified as a species of the genus *Flavobacterium*. It has been maintained by monthly transfer on a sea-water nutrient agar medium.

Inoculum media. Initially the inoculum medium used contained 0.8 per cent nutrient broth and 0.5 per cent yeast extract diluted to volume with artificial sea water. After requirements for growth in a chemically defined medium had been established, the basal medium was used as the inoculum medium. The experiments reported in this paper were conducted using the latter medium. Ten ml volumes of inoculum medium were dispensed into 50-ml Erlenmeyer flasks. Inoculum cultures were incubated 48 hr at 25 C on a rotary shaker. Inocula were washed by centrifuging the cells, removing the supernatant fluid and resuspending the cells in an inorganic salt solution containing the same salts at the same levels as were used in the basal medium. This operation was repeated twice more. A 1:100 dilution of the culture was made and one drop of this suspension (corresponding to about 10^6 organisms) was added per assay flask.

Basal medium.[1] The basal medium used in the early studies is shown in footnote 1. The medium employed to obtain the results reported here is shown in table 1. The most important difference between this medium and the one used initially lay in the replacement of the purine and pyrimidine bases by their corresponding nucleotides and the addition of a surface active agent Triton X-100 (a polyoxyethylene derivative of octyl phenol). Other changes included the use of a simplified salt mixture instead of artificial sea water, the addition of three vitamins instead of nine and the inclusion of tris(hydroxymethyl)-aminomethane (Tris) buffer. Ten ml volumes of the medium were dispensed into 50-ml Erlenmeyer flasks fitted with a tube on the side. The flasks were incubated at 25 C on a rotary shaker. Growth was measured as turbidity in an Evelyn colorimeter after various intervals of time, with the aid of the side tube attachment (MacLeod and Onofrey, 1956).

Chemicals used. The nucleosides and nucleotides used were obtained from the California Foundation for Biochemical Research. Synthetic di-myristoyl lecithin was generously supplied by Dr. Morris Kates, National Research Council, Ottawa. We are indebted to Dr. D. J. Hanahan, University of Washington, Seattle, for supplying egg lysolecithin and yeast lecithin purified by chromatography.

[1] Medium used in preliminary studies (in amounts per 100 ml of final medium): Glucose, 400 mg; $(NH_4)_2SO_4$, 100 mg; adenine, 1 mg; guanine, 1 mg; uracil, 1 mg; KH_2PO_4, 0.075 mg; KH_2PO_4, 0.075 mg; amino acid mixture (as in table 1), 10 ml; riboflavin, 100 μg; calcium pantothenate, 100 μg; thiamin, 100 μg; niacin, 100 μg; pyridoxal, 20 μg; *p*-aminobenzoic acid, 20 μg; biotin, 1 μg; folic acid, 1 μg; vitamin B_{12}, 0.15 μg; artificial sea water (MacLeod and Onofrey, 1956) as diluent.

FRB No. 521

TABLE 1

Basal medium for the growth of marine Flavobacterium B4

Component	(Amount Added per 100 ml final medium)
ucose	400 mg
$H_4)_2SO_4$	100 mg
ino acid mixture*	20 ml
enylic acid	7 mg
anylic acid	7 mg
tidylic acid	7 mg
iton X-100	500 μg
acin	480 μg
iamin	390 μg
otin	2 μg
Cl	2.24 g
$gCl_2 \cdot 6\ H_2O$	0.41 g
Cl_2	0.110 g
Cl	0.05 g
H_2PO_4	2.5 mg
HPO_4	2.5 mg
$SO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.7 mg
is(hydroxymethyl)amino methane†	600 mg

* Amino acid mixture: DL-alanine, 80 mg; -aspartic acid, 32 mg; L-glutamic acid, 40 mg; rginine hydrochloride, 16 mg; L-lysine hydro- loride, 16 mg; L-histidine, 8 mg; DL-isoleucine, mg; L-leucine, 4 mg; DL-methionine, 8 mg; -phenylalanine, 8 mg; L-proline, 4 mg; DL- reonine, 8 mg; L-tyrosine, 4 mg; DL-valine, mg; DL-tryptophan, 8 mg; L-cystine, 4 mg; -serine, 8 mg; and glycine, 4 mg.

† Adjusted to pH 7.0 with HCl.

Chromatographic purification of Tween 40. icic acid (Mallinckrodt 2847) was mixed in a 1 ratio with acid washed celite. A slurry of g of the mixture was made with $CHCl_3$ and ded to a column with an inside diameter of mm. Commercial Tween 40 (polyoxyethylene rbitan monopalmitate), 300 mg dissolved in HCl_3, was added to the column. $CHCl_3$, 3.5 per nt methanol in $CHCl_3$ and 10 per cent methanol $CHCl_3$ were added in successive 400-ml lumes. The eluates from the $CHCl_3$ and 3.5 per nt methanol in $CHCl_3$ were discarded while nt from the 10 per cent methanol in $CHCl_3$ was llected and evaporated to dryness. The residue d the appearance and surface active properties the original Tween except that the product s colorless instead of pale yellow.

RESULTS

Preliminary studies indicated that organism B4 failed to grow unless a supplement of natural materials was added to the basal medium (MacLeod *et al.*, 1954). Further investigation revealed, however, that growth would occur in the basal medium (footnote 1) if the incubation period were sufficiently long. The factor or factors concerned were therefore stimulatory rather than essential for the growth of the organism, although more extensive growth always occurred in their presence than in their absence.

Efforts were directed toward the fractionation and concentration of the active component or components in natural materials. From ion exchange chromatography and continuous electrophoresis a large number of fractions were obtained which stimulated growth of the Flavobacterium but not to the same extent as the unfractionated starting material. Since it was apparent that the various fractions contained compounds or groups of compounds differing significantly in chemical structure from fraction to fraction, it was suspected that the effect of these compounds on the organisms might be primarily physical rather than chemical in nature. Accordingly, a number of surface active agents were tested for growth-promoting activity. All stimulated growth of the organism when added to the unsupplemented medium but none to the same extent as yeast extract or other natural materials. Accordingly, various known growth factors were added to the medium in combination with a surface active agent. The only preparation found to increase the rate of growth of the Flavobacterium was an alkaline hydrolyzate of yeast ribose nucleic acid prepared by a procedure described by Loring *et al.* (1948). It was then observed that the ribonucleic acid (RNA) hydrolyzate could be replaced by a mixture of three nucleotides.

The relation of yeast extract, a surface active agent, and a mixture of three nucleotides, adenylic, guanylic, and uridylic acids, to the growth of the organism, is shown in table 2. In this experiment the medium of table 1 without nucleotides and Triton X-100 was used as the basal medium. It can be seen that growth occurred in the unsupplemented medium but only after a long incubation period. The addition of yeast extract reduced the time for a significant amount of growth to occur from 12 days to less than 2. Both Triton X-100 and the nucleotide mixture

TABLE 2

The effect of various supplements on the rate of growth of a marine Flavobacterium

Additions to Medium* (per 10 ml)	Incubation Time (hr)						
	49	67	96	115	139	216	288
	Light transmission (%)†						
None	100	100	100	100	100	94	52
Yeast extract (50 mg)	42	36	35	36	39	—	—
Triton X-100 (50 μg)	100	100	100	64	43	44	—
Adenylic + guanylic + uridylic acids (1 mg of each)	100	100	100	61	35	—	—
Nucleotides + Triton X-100	100	89	34	31	30	—	—

* The medium of table 1 from which the nucleotides and Triton X-100 had been omitted was used as the basal medium.

† Evelyn colorimeter readings, 660 mμ filter, uninoculated blank = 100.

added separately stimulated growth of the Flavobacterium but the two together were more active in this capacity than either alone. The combination, however, was somewhat less active than yeast extract in promoting growth.

The growth-promoting effect of some representative surface active agents tested is shown in table 3. All of these compounds promoted growth at low concentrations in the medium. It is interesting that even so potent an antibacterial agent as the quaternary salt benzyl myristyl dimethyl ammonium chloride had growth-promoting activity when tested at a low enough concentration. Tween 40 was found to be effective at low concentration only after purification. The procedure described by Davis (1947) for purifying Tween 80 was not successfully adapted for use with Tween 40, so the chromatographic procedure was applied. When commercial Tween 40 was used, levels ranging from 10 to 50 mg per flask were required to promote growth. After purification, table 3, 50 μg produced a maximum response. The difference in requirement for the commercial and purified Tween appears to be due to the presence of an inhibitor in the commercial product, since the commercial Tween inhibited growth when added to the medium in the presence of the purified material. As the component fatty acid of Tween 40, palmitic acid, was found to be nontoxic over the range of concentrations that could be tested, it is unlikely that this compound was responsible for the inhibitory effect.

TABLE 3

The response of a marine Flavobacterium to a number of surface active agents

Addition to Medium*	Amount of Agent (μg per 10 ml)			
	0	10	50	100
	Light transmission (%)†			
1. Cholic acid	100	100	100	31
2. Triton X-100	100	32	30	33
3. Span 40‡	100	85	31	34
4. Lecithin§	100	32	33	34
5. Tween 40¶	100	98	32	30
6. Benzyl myristyl dimethyl ammonium chloride	100	49	100	100
7. Digitonin	100	—	34	68

* The medium of table 1 was used with Triton X-100 omitted.

† Evelyn colorimeter readings, 660 mμ filter. Uninoculated blank = 100. Incubation times: Compounds 1, 2, and 3, 99 hr; 4 and 5, 112 hr; 6, 114 hr; 7, 123 hr.

‡ Sorbitan mono-palmitate.

§ Synthetic dimyristoyl lecithin.

¶ Purified by chromatography.

All of the even numbered saturated fatty acids from C_8 to C_{20}, oleic acid, and linoleic acid, were tested for growth-promoting activity. The longer chain fatty acids increased the rate of growth slightly over that occurring in flasks without added surface active agent but no more than would be expected from their capacity to act as relatively weak surface active agents. With the exception of lauric acid and to a lesser extent

TABLE 4

Nature of the response of a marine Flavobacterium to nucleotides

Additions to Medium*	Incubation Time (hr)					
	71	95	119	144	192	287
	Light transmission (%)†					
None	100	100	79	49	47	48
Adenylic acid	100	100	100	94	48	50
Guanylic acid	100	100	100	81	39	38
Uridylic acid	100	100	100	51	42	43
Adenylic + guanylic acid	100	100	100	99	67	52
Adenylic + uridylic acid	100	100	100	100	79	47
Guanylic + uridylic acid	100	100	100	81	34	34
Adenylic + guanylic + uridylic acids	65	34	36	37	—	—

* The medium of table 1 was used with the nucleotides omitted in its preparation. Each of the supplements was added at a level of 1 mg per 10 ml of medium as its Na^+ salt.

† Evelyn colorimeter readings, 660 mμ filter, uninoculated blank = 100.

caprylic and capric acid none of the acids proved toxic as evidenced by the fact that none of them delayed the appearance of growth over that occurring in parallel unsupplemented tubes. Sodium acetate was without effect on growth of the organism.

The nature of the response of the marine Flavobacterium to nucleotides is presented in table 4. The results show that any one or combination of two of the nucleotides added to the medium actually reduced the rate of growth. Only when all three were present in the medium together was an increase in the rate of growth obtained.

The specificity of the response to nucleotides is shown in table 5. Uridylic acid could be replaced in the mixture by uridine, cytidylic acid, or cytidine. The corresponding free pyrimidine bases were inactive. Deoxycytidine and thymidine, though ineffective in 96 hr, had some growth-promoting activity on longer incubation. Guanylic acid could be replaced equally well by guanosine or deoxyguanosine but not by guanine. Adenylic acid and adenosine could be used interchangeably but not adenine. Deoxyadenosine not only did not promote growth but prolonged the incubation period required to achieve growth considerably beyond that found necessary in the absence of the adenine nucleotide.

Other growth requirements. The organism was found to require both biotin and thiamin for growth (0.1 and 10 mμg per ml of medium, respec-

TABLE 5

Specificity of the response to nucleotides and related compounds

Addition to Medium*	Light Transmission (%)†
Adenylic + guanylic acids	
+ 0	100
+ uridylic acid	39
+ uridine	32
+ uracil	100
+ cytidylic acid	42
+ cytidine	36
+ deoxycytidine	99
+ thymidine	100
+ cytosine	100
Adenylic + uridylic acids	
+ 0	100
+ guanylic acid	30
+ guanosine	32
+ deoxyguanosine	32
+ guanine	100
Guanylic + uridylic acids	
+ 0	100
+ adenylic acid	41
+ adenosine	41
+ deoxyadenosine	100
+ adenine	100

* The medium of table 1 with the nucleotides omitted was used as the basal medium. Nucleotides added as their Na^+ salts where shown.

† Evelyn colorimeter readings, 660 mμ filter. Uninoculated medium = 100. Incubation time, 96 hr.

tively, for maximum response). Niacin was originally thought to be stimulatory and was included in the medium, table 1. In the final medium, however, a requirement for niacin could not be demonstrated.

When amino acids were left out of the mixture one at a time, requirements for 6: isoleucine, leucine, valine, arginine, methionine and glycine, were observed. Removal of amino acids similar in structure, 2 at a time, from the medium revealed that neither leucine nor isoleucine was required if both were omitted together. Growth would not take place if both aspartic and glutamic acids were omitted. No simple combinations of required and stimulatory amino acids tested permitted growth of B4. For this reason the complete mixture of 18 amino acids was included in the final medium. Glucose could not be shown to be either required or stimulatory for growth of the organism in this medium.

The Flavobacterium was found to have a requirement for Na^+, K^+, Mg^{++}, and Ca^{++} for growth.

DISCUSSION

There seem to be few reports of the growth-promoting action of surface active agents for bacteria where the effects observed can be clearly ascribed to the surface active properties of the compounds concerned. In the case of a number of lactic acid bacteria which responded to the addition of Tweens to the medium, the organisms were shown to require oleic or linoleic acid for growth. The ability of the Tweens to replace these compounds in the medium in these experiments could be attributed to the capacity of the Tweens to serve as nontoxic sources of oleic acid (Williams *et al.*, 1947). When a variety of lipids and lipid derivatives, some of which were surface active agents, were found to be active in promoting growth of bacteria, it was postulated that the compounds were physiologically related for the organisms through a capacity to act as precursors of cell lipids (Guirard *et al.*, 1946). In the present study a variety of surface active compounds differing widely in structure have been found to have growth-promoting activity. One of the most active of these, Triton X-100, is not a lipid derivative and hence would not be expected to be physiologically related to the other compounds through a capacity to act as a precursor of cellular lipids. Furthermore, fatty acids had no specia growth-promoting properties for the organism It therefore seems reasonable to conclude tha surface activity is the property of the surface active agents tested which is responsible for thei growth-stimulating effect on this organism.

In the case of Mycobacteria the addition of a surface active agent was responsible for produc ing an even, well-dispersed culture in liquic media (Dubos and Davis, 1946). For the Flavo bacterium reported here, clumpy, poorly dis persed growth occurred even in the presence of a surface active agent unless the nucleotide com bination was present in the medium. The pres ence or absence of surface active agents in the medium appeared to have no effect on the degree of dispersion of the culture.

There have been numerous reports of nucleo tides and nucleosides being essential or stimu latory for the growth of microorganisms, (Loring and Pierce, 1944; Kitay *et al.*, 1949; Hoffman and Pavcek, 1952). In all of these cases single nucleo sides or nucleotides had growth-promoting activity, though combinations were frequently more effective than individual nucleotides tested alone. The findings reported here appear to be unique in that the individual nucleotides tested alone or in pairs are inhibitory. Only when the three are present together does stimulation of growth result. These findings appear to be analogous to the complicated interrelationships found to exist among certain of the amino acids, for instance, leucine, isoleucine, and valine (Tomlinson and MacLeod, 1957). Since guanosine and deoxyguanosine are interchangeable in the mixture and since both would be needed for growth by the organism, imbalances in the medium in this case would most probably be inhibiting growth by affecting nucleotide synthesis. The capacity of guanosine and deoxyguanosine to replace each other would thus indicate that the two compounds are interconvertible by the cell either directly or through a common precurso with which they are both in equilibrium. This conclusion is of interest since little is known o the origin of the deoxynucleosides or nucleotides Reichard (1955) has presented evidence indicat ing that uridine is converted to thymidine vi deoxyuridine in the rat. McNutt (1957) in studie with a Neurospora mutant has concluded tha the carbon skeleton of the purine deoxyriboside

as as its origin the intact carbon skeleton of the urine ribosides. The findings reported here suggest that not only can ribose nucleosides serve as precursors for the deoxy compounds but that the reactions involved are readily reversible.

The complexity of the growth requirements of his marine Flavobacterium approaches that of he most fastidious bacteria studied found growng on land (Knight, 1945). The phytoplankton ith which most of the marine bacteria in the sea re associated (ZoBell, 1946) could be expected serve as a source of the nutrients required by is organism. Despite their complexity, there is othing unique about the nature of these organic owth requirements. Only in having a requirement for Na^+ can this marine Flavobacterium ke the other marine bacteria studied in this boratory, at the present state of knowledge, be stinguished from land forms (MacLeod and nofrey, 1957).

SUMMARY

A marine organism of the genus *Flavobacterium* hich grew very slowly in a chemically defined edium unless yeast extract or other natural aterials were added responded almost as well a combination of a surface active agent and ree nucleotides as to yeast extract.

It was concluded that the surface active compunds promoted growth because of their surface tive properties rather than for any capacity to rve either as precursors of cell lipids or as urces of fatty acids.

Adenylic, guanylic, and either uridylic or cytilic acids, when tested together, stimulated owth of the Flavobacterium although any one combination of two of the nucleotides added the medium actually reduced the rate of owth of the organism. Uridylic acid could be placed in the mixture by uridine, cytidylic acid, cytidine. Deoxycytidine and thymidine, ough less effective, had some growth-promoting tivity. The free pyrimidine bases were inactive. uanylic acid could be replaced equally well by anosine or deoxyguanosine but not by guanine the combination. Adenylic acid and adenosine uld be used interchangeably but not adenine. eoxyadenosine proved to be inhibitory.

It was concluded that guanosine and deoxyanosine and possibly other ribosides and their corresponding deoxy compounds are interconvertible by this organism.

The Flavobacterium was found also to require biotin, thiamin, a complex mixture of amino acids, Na^+, K^+, Mg^{++}, and Ca^{++} for growth.

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THE ACID-SOLUBLE NUCLEOTIDES OF SALMON LIVER[1]

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Abstract

The acid-soluble nucleotides of spring salmon liver have been separated by anion-exchange chromatography at low temperature and characterized. Under these conditions the relatively labile uridine-5′-diphosphate nucleotides of acetylglucosamine, galactose, and glucuronic acid were obtained intact, a fact that is further substantiated by the complete absence of uridine-5′-diphosphate. The occurrence of these uridine diphosphate compounds and the absence of uridine diphosphate glucose is discussed in relation to the previously postulated role of inositol as a carbohydrate storage product. A new peptide-containing nucleotide succinoadenosine-5′-phosphosulphate (peptide), was found in the fraction which immediately follows adenosine-5′-diphosphate. The parent base of this nucleotide, succinoadenine, was also isolated. The nucleotide pattern is simpler than that reported by other investigators for rat liver and wheat.

Introduction

Since the application of anion-exchange chromatography by Cohn (6, 7) to the separation of nucleotides, Hurlbert *et al.* (16) have applied this method to the separation of the acid-soluble nucleotides of rat liver. In recent years extensive work has been carried out on the identification and coenzymic role of nucleotides on a variety of tissues too numerous to review. However, very few published reports have appeared concerning the nucleotides in marine life. Jones and Murray (18) reported the presence of ATP,* ADP, AMP, DPN, IMP for rested and exhausted skeletal muscle of the codling. Storey and Love (36) partially characterized a succinoadenylic acid-like compound from cod liver, and Tsuyuki and Idler (39) identified a peptide of 6-succinoadenosine-5′-phosphosulphate in salmon liver.

In this study the acid-soluble nucleotide pattern of salmon liver will be presented and compared with the results obtained by other investigators. In addition, the advantages of low temperature chromatography employed in this study will be discussed.

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*The following abbreviations are used in this manuscript: UMP, uridine-5′-phosphate; UDP, uridine-5′-diphosphate; UTP, uridine-5′-triphosphate; AMP, adenosine-5′-phosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; IMP, inosine-5′-phosphate; IDP, inosine-5′-diphosphate; ITP, inosine-5′-triphosphate; GMP, guanosine-5′-phosphate; GDP, guanosine-5′-diphosphate; GTP, guanosine-5′-triphosphate; DPN, diphosphopyridine nucleotide; UDPAG, uridine-5′-diphosphate acetylglucosamine; UDPGAL, uridine-5′-diphosphate galactose; UDPGA, uridine-5′-diphosphate glucuronic acid; UDPX, uridine-5′-diphosphate derivative where X is a variable; P_t, total phosphorus; P_l, acid-labile phosphorus.

Methods

Preparation of Acid-soluble Fractions

The livers were excised from live spring salmon (*Oncorhynchus tschawytscha*) at sea and immediately frozen in dry ice. The powdered tissue was homogenized in a precooled Waring blendor with cold 0.6 *N* perchloric acid (1.5 ml. per g. of tissue) and centrifuged. The residue was extracted a second time with the same volume of cold 0.2 *N* perchloric acid. The supernatants were combined and neutralized with 5 *N* potassium hydroxide to pH 7.0. All operations were carried out at 0° C. The slightly soluble potassium perchlorate (0.75 g. per 100 ml. at 0° C.) began to precipitate immediately, and after standing a few hours the supernatant was decanted, lyophilized to one-quarter its volume, and a further crop of potassium perchlorate removed to reduce the salt concentration. The sample was chromatographed on Dowex-1 anion-exchange resin employing gradient elution exactly as described by Hurlbert *et al.* (16). The liver nucleotides were separated into fractions A to J by elution with formic acid. Each of these fractions was then further purified on Dowex-1, using an ammonium formate – formic acid solvent system.

Low Temperature Chromatography

When the nucleotide pattern from a column operated at room temperature according to Hurlbert *et al.* (16) was compared to that obtained at 0° C., the differences observed warranted the use of refrigeration throughout the chromatographic procedures. The Gilson fraction collector pan was insulated to minimize heat exchange and the refrigerant was circulated through the pan and the jacketed column at a rate sufficient to maintain the temperature of each at 0° C.

Analysis and Rechromatography

The nucleotide fractions were initially located by reading their optical densities at 260 mμ in a Beckman spectrophotometer. Subsequently, the nucleotides were located automatically by means of an ultraviolet recorder attached to the Gilson fraction collector. In this system a combination of filters permitted ultraviolet light in the region of 257 mμ to pass through a quartz cell through which the eluent passed. Thus only material absorbing in the 257 mμ region is recorded. When the results from several column runs obtained by the automatic ultraviolet recording device were compared to the readings from the Beckman DU, it was found that with the exception of a few very small components all major and minor peaks were detected.

Appropriate fractions of a peak were pooled and the solvents were removed by freeze-drying and the nucleotides analyzed or again chromatographed. The ammonium formate was removed by several methods; (*a*) by freeze-drying for several days; (*b*) by ion-exchange (IR-120) followed by freeze-drying; and (*c*) by freeze-drying followed by high vacuum (10^{-7} mm. Hg) sublimation for a few hours. The samples were then dissolved in water and aliquots taken for the various analyses.

Analytical Methods

Ultraviolet Light

The maximum and minimum ultraviolet absorption of the nucleotides in acid (pH 1-2) was read from a Beckman Model DK spectrophotometer recording. The nucleotide concentrations in acid were calculated from the appropriate extinction values compiled by the California Foundation for Biochemical Research. Adenine succinic acid was determined by absorption at 266 mμ, using $E = 16.9 \times 10^3$ at pH 1.0.

Phosphate

Inorganic phosphate was determined by the method of Lowry and Lopez (24). All recorded values for total and labile phosphate have been corrected for inorganic phosphorus. For labile phosphate the sample was hydrolyzed directly in 18×150 mm. calibrated colorimeter tubes, containing 4.0 ml. of 1 *N* sulphuric acid, for 15 minutes in a boiling water bath. The color was developed by the Fiske-Subbarow method (11) and read after 15 minutes in an Evelyn colorimeter with a 660 mμ filter. For total phosphorus the samples were brought to dryness in colorimeter tubes and digested with 1.0 ml. of 4 *N* perchloric acid for at least one hour at 160° C. (20). The tubes were then partially cooled, one drop of 30% hydrogen peroxide was added, and the contents were digested for an additional 40 minutes. The color was developed as for labile phosphate.

The position of the phosphate attached to the nucleotide sugar was determined by spraying the paper chromatograms with di-(*p*-dimethylaniline) methane reagent (41) as well as by use of the specific enzymes, 3′- and 5′-nucleotidase.

cis-Glycol and Sulphate

cis-Glycol was determined spectrophotometrically (9). Sulphate was determined colorimetrically after conversion to hydrogen sulphide (25), which was then reacted with *p*-phenylenediamine to form Lauth's violet (32).

Sugars

Ribose was determined according to Meijbaum (26). The ribose values of pyrimidine ribosides were used qualitatively. Uronic acid was determined by the Dische (8) method, reducing sugars by the Folin and Malmros (12) method, and N-acetylglucosamine by the method of Reissig *et al.* (33). In the Reissig assay the large difference in the extinction values enabled qualitative distinction between N-acetylglucosamine and N-acetylgalactosamine.

Amino Acid

The peptides were hydrolyzed in 6 *N* hydrochloric acid for 12 hours at 120° C. in sealed tubes. The acid was removed at 100° C. in a stream of nitrogen and the amino acids were determined quantitatively by the method of Moore and Stein (27).

Paper Chromatography

Whatman No. 1 and occasionally No. 3 papers were used ascending. Where it was necessary to elute the nucleotides for further analyses washed papers were used because of ultraviolet-absorbing contaminants in the paper. The papers were washed with a 1:1 mixture of ethanol and 1.2 *N* acetic acid, and rinsed thoroughly with water followed by ethanol according to the procedure of Kay *et al.* (19). The solvent systems used are indicated in the tables. The free bases and their derivatives were located by use of a mineralight lamp, the hexoses and uronic acid by aniline phthalate (31), hexosamines and acetylhexosamines by the modified Elson-Morgan reagent (30), amino acids by ninhydrin (22), and phosphorus by the Hanes and Isherwood method (14). Both the purine and pyrimidine nucleotides were hydrolyzed to their free bases by heating for 2 hours at 175° C. with 90% formic acid.

Results

A typical chromatogram of the acid-soluble nucleotides of salmon liver is shown in Fig. 1. Fractions A to J inclusive were present in every column that was analyzed. No fraction appeared beyond J even after prolonged elution. However, the relative amounts of each fraction showed considerable variation between individual salmon and between sexes. Each of the fractions has been analyzed and its principal components have been characterized.

The properties of fractions A, B, C, D, H, and J are summarized in Table I, together with the analytical data for their identification. Fraction A was separated into three components (A_1, A_2, and A_3) by column chromatography using ammonium formate as the developer. When each of these was subjected to further separation on paper, A_1 proved to be a single component, DPN, showing a typical cyanide reaction. A_2 was composed of two fractions, A_{2a} and A_{2b}, which were AMP and succinyl adenine respectively. These two components behave chromatographically alike in many systems but could be separated in *n*-butanol – acetic acid. A_3 was also separated into two components on paper. A_{3a} exhibited the ultraviolet absorption of uric acid. However, uricase did not reduce the absorption at 283 mμ and it was distinguishable from uric acid by paper chromatography using *n*-propanol – water (60:40). A_{3b} has not yet been identified.

Peak B proved to be a single component, GMP.

After a second column purification fraction C was separated into six components (Fig. 2). However, during the initial stages of this investigation, when the ion-exchange procedure was carried out at room temperature, it is significant that only two components (UMP and IMP) were obtained. Presumably the less stable remaining components were destroyed during column chromatography. The first component, C_1, proved to be hypoxanthine. When the known hypoxanthine derivatives IMP, IDP, and ITP were treated with 4 *N* formic acid for 3 days at room temperature (conditions considerably more drastic than the chromatographic procedures) there was no evidence of free hypoxanthine. At present there is no explanation for the presence of

hypoxanthine in this fraction except as a product of the partial decomposition of one of the C fractions C_4, C_5, or C_6. Components C_2 and C_3 were shown to be UMP and IMP respectively. C_4 consists of the bases uracil and hypoxanthine. The identification of C_5 and C_6 is continuing.

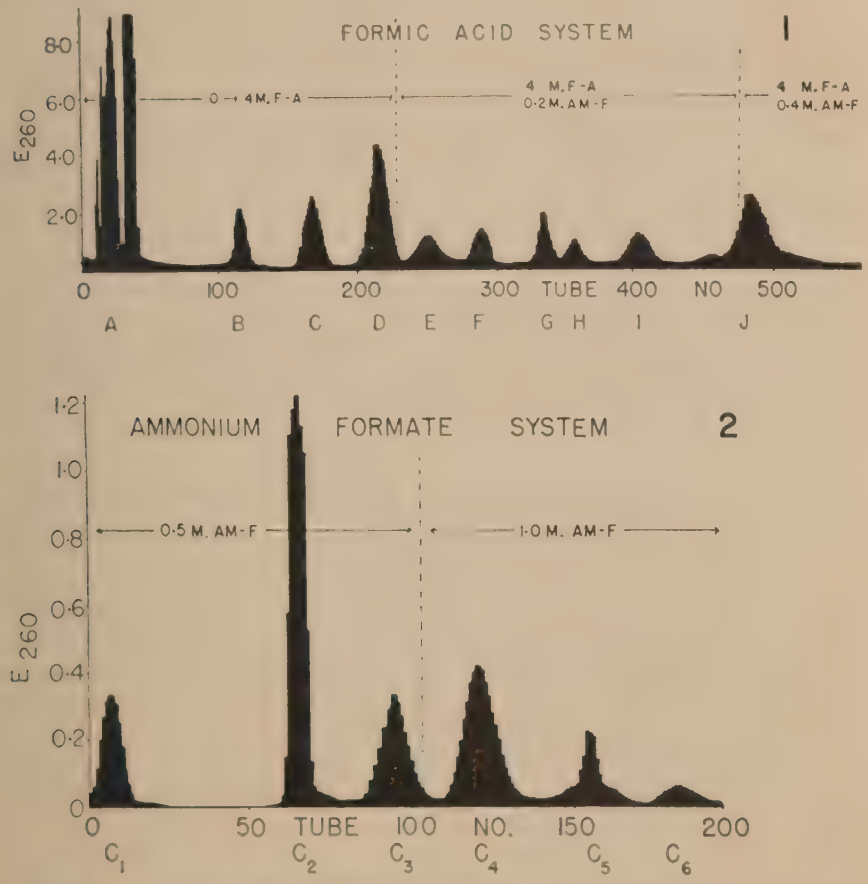


FIG. 1. Chromatography of the perchloric acid extract from 125 g. of liver using the formic acid system. The nucleotides were separated by gradient elution on a 1.7×28 cm. bed of Dowex-1 formate resin at 0° C. The mixing volume was 2 l. and 10 ml. fractions were collected. The optical densities of the eluted fractions at 260 mμ are plotted for each chromatographic fraction. The symbol F.A. refers to formic acid and AM-F to ammonium formate.

FIG. 2. Rechromatography of fraction C. Fraction C obtained from the formic acid system was rechromatographed on a 1×20 cm. bed of Dowex-1 formate resin at 0° C. The mixing volume was 500 ml. and 5.0 ml. fractions were collected. The column was developed with an ammonium formate solution.

TABLE I

ANALYSES OF FRACTIONS A, B, C, D, H, AND J

Fraction	Components	Chromatographic properties similar to:*	Spectra in acid		Per mole			Base*
			λ max.	λ min.	P_t	P_i	Ribose	
A	A_1	DPN[1,7]	256	236	1.82	0		Adenine[4,6]
	A_{2a}	AMP[1,7]	257	230	0.88	0	0.91	Adenine[4,6]
	A_{2b}	Succinoadenine[1,7]	270	231	0	–		
	A_{3a}[7]		283		0			
	A_{3b}[7]		257					
B	B_a	GMP[1,7]	257	228	1.04	0	1.20	Guanine[4,6]
C	C_1	Hypoxanthine[1,4,6]	248	215	—	—	—	Hypoxanthine[1,4,6]
	C_2	UMP[1,7,9]	261	230	0.93	0	+	Uracil[4,6]
	C_3	IMP[1,7,9]	248	224	1.04	0	1.0	Hypoxanthine[1,4,6]
	C_4							Uracil and hypoxanthine
	C_6		256	231				Adenine[4,6]
D	D	ADP[1,8]	257	231	2.0	0.92	1.0	Adenine[1,4,6]
H	H	GDP[1,8]	257	230	2.0	1.06	—	Guanine[4,5]
J	J	ATP[1,8]	257	236	3.1	2.2	–	Adenine[4,6]

*Superscripts refer to solvent systems used as follows:
(1) Isobutyric acid/conc. ammonium hydroxide/water, 66/1/33; (2) ethanol/1 *M* ammonium acetate (pH 3.8), 7.5/3; (3) 0.1 *M* sodium phosphate, pH 6.8/ammonium sulphate/*n*-propanol, 100/60/2; (4) aqueous ammonia, pH 10.0; (5) *n*-butanol/water/formic acid, 77/13/10; (6) water-saturated *n*-butanol/ammonia, 100/1; (7) *n*-butanol/acetic acid/water, 2/1/1; (8) isobutyric acid/conc. ammonium hydroxide/water, 57/4/39; (9) ethanol 1 *M* ammonium acetate (pH 4.5), 65/35; (10) ethyl acetate/pyridine/ammonium hydroxide/water, 40/20/12/12, paper treated with boric acid; (11) *n*-butanol/acetic acid/water (boric acid saturated), 4/1/5; (12) *n*-butanol/pyridine/water, 45/25/40.

Fraction D consisted solely of ADP, H of GDP, and J of ATP (Table I).

Fractions F, G, and I are all derived from UDP and have been identified as UDPAG, UDPGAL, and UDPGA respectively. Whereas UDPAG has been isolated and characterized from yeast (29, 3), animal liver (28, 15), hen oviduct (37), mung bean seedlings (34), and UDPGAL from mung bean seedlings (13), and UDPGA in rabbit liver (35), and mung bean seedlings (34), their presence in marine forms of life has not yet been reported. The analytical data on which the identification of F, G, and I is based appear in Table II. The characterization of UDPAG is based on a molar ratio of base: P_t:P_i: reducing value: N-acetylglucosamine of 1:2:1:1:1. N-Acetylglucosamine was identified by paper chromatography and by colorimetric assay (33), and differs from N-acetylgalactosamine by having an extinction value about three times greater at 585 mμ.

TABLE II

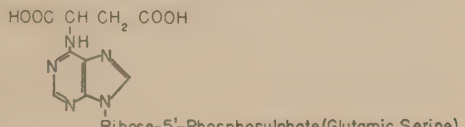
PROPERTIES OF UDPX COMPOUNDS IN SALMON LIVER

Fraction*	Chromatographic properties similar to†	Base†	Per mole			Reducing value	Uronic acid	N-Acetyl glucosamine	X component†
			P_t	P_i	Ribose				
F	UDPAG[1,2,3]	Uracil[4,6]	1.88	0.92	+	1.08		1.05	N-Acetyl-glucosamine[2,10]
G	UDPGAL[1,2,3]	Uracil[4,5]	2.05	0.92	+	1.0			Galactose[11,12]
I	UDPGA	Uracil[4,5]	1.97	1.01	+	+	0.86		Glucuronic[7,9] acid

*All three fractions showed a uridine type spectrum.
†Superscripts refer to solvent systems in Table I.

Fraction G was identified as UDPGAL by virtue of equimolar proportions of UDP and reducing sugar. The sugar was shown to be galactose on several paper chromatograms. Fraction I was likewise shown to be UDPGA by a UDP to uronic acid ratio of approximately unity. Glucuronic acid was distinguished from galacturonic acid by paper chromatography and by the ease with which the former compound lactonizes (30).

A hitherto unknown nucleotide, of which a preliminary account has appeared (39), was found in the E fraction, which immediately follows ADP in the chromatographic picture (Fig. 1). This fraction was separable into three components (E_1, E_2, and E_3) by paper chromatography. Compound I (E_2) moved between the other two and generally comprised approximately 80% of the fraction. The minor components E_1 and E_3 are still being investigated. Analytical data on I show an approximately equimolar ratio of succinoadenine, total P, ribose, sulphate, and *cis*-glycol (Table III). A peptide consisting of glutamic acid and serine was found to be associated with this compound. On the basis of these analyses and partial degradation studies (39) the structure indicated below has been tentatively assigned to this compound.



Compound I

TABLE III

ANALYSIS OF COMPOUND I

	Moles
Succinoadenine*	1.00
Ribose	1.12
Labile P	0.00
Total P	1.05
Sulphate	1.37
cis-Glycol	1.27

*Arbitrarily chosen as 1 mole.

The occurrence of either succinoadenine or succinoadenylic acid has been reported in yeast (4), *Penicillium chrysogenum* (1), *Escherichia coli* (23), mammalian liver (17), and cod liver (36). The lability of compound I suggests the possibility that succinoadenylic acid isolated from other natural products by ion-exchange chromatography at room temperature is a decomposition product from a larger molecule. The apparent absence of adenyl succinic acid in the present study strengthens this belief. The occurrence of succinoadenine in fraction A appears to be in disagreement with Joklik's statement (17) that it behaves chromatographically similar to succinoadenylic acid.

Discussion

The distribution of acid-soluble nucleotides in fish liver is different from that of other tissues. Whereas in rat liver (16) and in wheat plants (2) the mono-, di-, and tri-phosphates of cytidine are present, these were not detected in the fish liver, nor were GTP, UDP, and UTP. The nucleotide picture appears to tend towards simplicity compared to that of the other two tissues. The absence of UDP, which should appear in the region of fractions F, G, and I, is significant. This nucleotide was not detected, though it was searched for specifically. It would appear then that unless low temperature column chromatography is employed UDP appears as a decomposition product of relatively labile UDPX compounds.

The role of UDPX compounds is interesting to consider. Fischer (10) hypothesized that the presence of relatively large amounts of inositol in shark fins and the absence of glycogen and other reserve carbohydrates in the liver might mean that inositol may function as a source of blood sugar by the re-opening of the inositol ring. Recently, in his biochemical studies on inositol, Charalampous (5) discovered an enzyme system from rat kidney which catalyzed the oxidative conversion of inositol to glucuronic acid through the postulated mediation of UDP glucose followed by UDPGA. An analysis of a number of fish livers in this laboratory showed that there is in this tissue a set of conditions suggestive of the role of inositol as a carbohydrate storage product. Inositol analyses from a number of tissues from salmon, including the liver, indicate the presence of this compound at relatively high levels (40). The presence of reducing sugars in fish tissue appears to be a post-mortem phenomenon; these compounds do not appear to be present in fresh fish (38). UDPGAL and UDPGA were found to be present in significant concentrations in the liver, whereas UDP glucose was found to be absent. The reversible conversion of UDP glucose to UDPGAL has been demonstrated by Leloir (21) in *S. fragilis*. UDPGAL and UDPGA, therefore, might be implicated in the conversion of inositol to glucuronic acid in addition to other coenzymic roles in which these nucleotides are involved.

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Sodium Phosphate Crystals on Salt Fish

Several times in the past few years, samples of undried or partly dried salt cod with an accumulation of crystals appearing over the surface have been submitted to this laboratory for examination. These deposits did not resemble either the sodium chloride or the calcium sulphate crystals often found on salt fish. One sample had many water-clear rhombic crystals with edges 2 cm. or more long. Others had a second type of crystals: they were dry, powdery white, and thickly scattered over the surface. Curiously, chemical analysis indicated that both types of crystals contained large proportions of phosphate and sodium, a small amount of chloride, but no sulphate, calcium or magnesium.

When the clear rhombic crystals were exposed to the dry laboratory atmosphere for a day, they changed into a white powder, similar to the second type. This powder contained 20·0 per cent moisture and appeared to be the dihydrate of disodium hydrogen phosphate. On analysis of the sample dried at 105° C., the sodium, phosphate and chloride contents indicated 96·0 per cent disodium hydrogen phosphate with 3·0 per cent sodium chloride.

Thus, the clear rhombic crystals appear to have been the dodecahydrate, the least soluble of the sodium phosphates, which in a dry atmosphere loses water to form the dihydrate (containing 20·2 per cent water) in a white powdery form. This is the form found in samples of the second type.

Since sufficient observations on the solubility of disodium hydrogen phosphate in a solution saturated with sodium chloride were not available, determinations were made at temperatures varying from 0° to 20° C. The results, with some values taken from the literature[1,2], are given in Table 1, and in Figs. 1 and 2.

Fishery salt contains only traces of phosphate. On the other hand, and in agreement with other results[3,4], fresh cod muscle was found to contain 0·2 per cent total phosphorus, of which 0·16–0·17 per cent is inorganic phosphate. Assuming a moisture content of 81 per cent, this would correspond with a concentration of 0·97–1·13 gm. anhydrous disodium phosphate per 100 gm. water. In undried salt fish this figure should remain unchanged: only dry salt is added during salting, and the concentration of phosphate in the water held by the fish should

Table 1

Temperature (° C.)	System $Na_2HPO_4.12H_2O$–NaCl–H_2O Solubilities (gm./100 gm. H_2O)						System $Na_2HPO_4.12H_2O$–H_2O Solubilities (gm./100 gm. H_2O)		
	Na_2HPO_4			NaCl			Na_2HPO_4		
	A	*B*	Average	*A*	*B*	Average	*A*	*B*	Average
0·3	0·579	0·586	0·583	35·41	35·22	35·31	1·76	1·73	1·75
2·5	0·669	0·658	0·664	35·68	35·67	35·68	1·96	1·97	1·97
4·8	0·899	0·900	0·900	35·67	35·60	35·64	2·28	2·28	2·28
10·1	1·77	1·77	1·77	35·40	35·36	35·38	3·60	3·26	3·43
14·5	3·09	3·11	3·10	34·79	34·87	34·83	4·82	4·82	4·82
21·6	7·11	7·08	7·10	33·44	33·41	33·43	8·14	8·15	8·15

Values *A*, equilibrium approached from higher temperatures; values *B*, equilibrium approached from lower temperatures

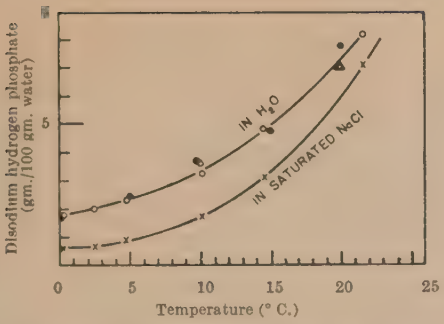

Fig. 1. Solubility of disodium hydrogen phosphate dodecahydrate in water and in saturated sodium chloride solution from 0° to 20° C. ●, In water, data from I.C.T. (ref. 1); ○, in water, experimental; ×, in saturated sodium chloride solution, experimental; △, in saturated sodium chloride solution (ref. 2)

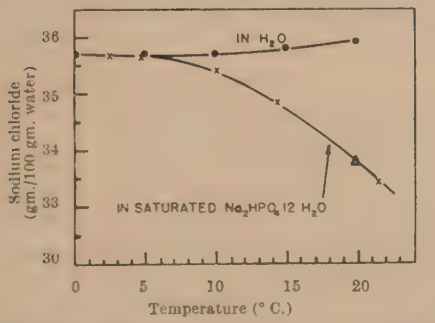

Fig. 2. Solubility of sodium chloride in water and in saturated disodium hydrogen phosphate dodecahydrate solution from 0° to 20° C. ●, In water, data from I.C.T. (ref. 1); ×, in saturated phosphate solution, experimental; △, in saturated phosphate solution (ref. 2)

be the same as in the pickle lost, unless some phosphate is held by the tissue. Such a solution, saturated with sodium chloride, would also be saturated with the disodium phosphate dodecahydrate at about 4° C.

After partial drying, the temperature of saturation with respect to the phosphate would be higher. In one sample of slightly dried heavy-salt fish, analysis for moisture and phosphate indicated that this temperature would be about 11° C. Drying therefore favours the formation of the crystals.

A slightly alkaline *p*H (about 7·5–8) is also required for the formation of disodium hydrogen phosphate crystals. While the fish itself is almost neutral, most solar fishery salts are alkaline, and any slight spoilage

(which often occurs with medium-salt fish) would also tend to increase the *p*H.

Thus the occurrence of these crystals on partially dried salt bulk fish, particularly while in cool storage or during transportation in the winter months, is readily explained. Its higher incidence with medium-cure fish probably depends on the susceptibility to rapid spoilage and the consequent elevation of the *p*H in this type of salt fish.

The effect of the sodium phosphate on the solubility of sodium chloride should also be noted. At 20° C. the solubility of the latter is only 93·9 per cent of the value in the absence of the phosphate. This explains why, in practice, salt fish is not often found in which the water is completely saturated with respect to sodium chloride when the solubility in salt-water systems only is considered, as is usually done.

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Nov. 8.

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Preservation of fresh fish

by

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Those whose lot it is to handle fish are only too familiar with its highly perishable nature. In this commodity we have a highly nutritious food which is, unfortunately, as valuable a nutrient for bacteria which occasion fish spoilage as it is for man himself. Consequently man has continually waged war against the spoilage microorganisms, and in this fight he has employed two general avenues of attack, namely, application of physical and chemical agents, or combinations of these. It is my purpose to try to give you a logical picture of the subject of spoilage of fresh fish and its control, with particular emphasis on the comparative value of various methods whereby it may be delayed. Seafoods which have been properly preserved from attack by microorganisms by canning, freezing and dehydrative procedures will not be considered.

Physical conditions

I think that it is correct to say that the early work of Hess (1) and of Huntsman (2) in Canada, which commenced about 1928, was responsible for calling attention to the fact that the rate of bacterial spoilage of fish was about twice as rapid at 2.5° C. as at −1° C. This discovery paved the way for all subsequent work which has been carried out in attempts to devise practical methods for storing fish at —1° C. from the time of capture until they are processed. It is obvious that fish, unless covered by a water-vapour impermeable material, cannot be stored in air at —1° C. without surface drying, if the atmosphere is not saturated with respect to water vapour. It is almost impossible to accomplish this last-named condition in practice for cooling surfaces automatically remove water from the atmosphere and therefore methods of storing fish at about —1° C. have either employed ices or dilute solutions containing various solutes which lower the freezing point. Probably one of the greatest values of ice lies in its ability to bathe surfaces of fish with a slow stream of clean water and thus help to remove bacteria and their metabolism products.

Lowering temperatures by use of special ices

Early attempts were made to lower the temperature of fish stored in melting ice by the simple expedient of scattering ordinary salt over the surface. This method was not satisfactory since there was almost invariably uneven distrubition of the salt. Heiss and Cursiefin (3) employed ices containing eutectic concentrations of disodium phosphate and sodium carbonate which had melting points of —0.9° C. and —2° C. respectively. Some improvements in keeping quality of fish iced with these ices rather than with ordinary water ice were observed. As far as can be ascertained, ices of this type were never used commercially, possibly because of their comparatively high costs. Early attempts to use ordinary salt as a cheap solute for this purpose were not successful because the high eutectic concentration of the salt caused it to migrate to the centre of ice blocks. Newer ice-making techniques which involve rapid freezing of water in

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comparatively thin flakes have enabled "sea-water ice" to be prepared. A recent report by Hansen (4) has shown that ices containing 1 % and 3 % of sea salt are slightly more effective than fresh-water ice in preserving iced cod, but slight salty flavours were sometimes noted in the fish stored in the ice containing the high concentration of salt. This method would still appear to offer possibilities, especially in localities in which there is a poor supply of fresh water. However, the corrosive nature of salt solutions makes the use of corrosion-resistant metals in the freezing equipment almost mandatory.

Refrigerated sea water

As has been pointed out in a recent popular review of this subject (5) the origin of this method is a little obscure, but there seems little doubt that the basic Canadian observations referred to previously were largely or entirely responsible for developments in this field. It was pointed out in our laboratories in 1947 (6) that the method of holding fish in refrigerated sea water at —1° C on vessels offered promise. However, it was not until about two decades after the early work of Huntsman and Hess that small-scale trials were made on trolling boats as a result of efforts of investigators at our Station (7, 8). The results obtained were very promising, and, in consequence, the work was expanded to a salmon-packing vessel capable of holding 40,000 pounds of fish, and more recently to a 75-ft. halibut vessel which is presently fishing for halibut in the Gulf of Alaska. Several large fishing companies on the West Coast of the United States have followed our lead and have already surpassed Canada in the number and size of refrigerated sea-water installations in active use. These installations have so far mainly been large scows (barges) for transporting salmon to canneries, or holding tanks for salmon on shore.

In British Columbia at the present time there is every indication that our initial experiments have favourably influenced fishermen, vessel owners and fish processors in their attitude toward use of this storage medium. Thus, a number of salmon trolling boats have installed chilling tanks; a new, large steel halibut long liner and seiner is being equipped to use this system exclusively, and another similar vessel is planned. Shore holding tanks intended for storing halibut and salmon intended for freezing or canning during times of fishing surplus are being built at three large British Columbia fish plants. Similar installations were used last year in Alaska. What are the advantages and disadvantages of this medium for storage?

In early work where tanks were installed in a compartment in fish-holding tanks in small vessels, there was the ever-present danger of galvanic action and attendant corrosion. As a result, the tanks, coils, etc. had to be constructed of corrosion-resistant aluminium or coated with an epoxy resin, "Lithcote", or other baked-on resin type of paint. In recent so far unpublished work (9) our engineers have shown that more rapid chilling of the sea water is attained if it is pumped quite rapidly through heat-exchange units. These are constructed of a polyethylene type of large diameter tubing and contain a bank of copper cooling coils, properly baffled and with aluminium extruders to facilitate heat transfer from the refrigerant to the copper surfaces. The expense of such a modified installation is very considerably less than previous ones based on the coil-in-tank method, and this fact will enable new vessels to employ this method much more economically.

There are many distinct advantages to this technique. The labour of icing fish is dispensed with and the eviscerated fish may be merely thrown into the tanks of sea water previously chilled to —1° C. where they cool quite rapidly. It is possible to keep uneviscerated fish several days in this medium under certain conditions, but the fish

must not have fed recently so that the enzymic and putrefactive activity in the visceral tracts is minimal. If the tanks on a vessel are properly filled into the "trunks", which usually extend slightly above deck, there is no danger of splashing and sudden shifting of fish. In fact, vessels equipped with these tanks handle very well in heavy seas. Unloading of fish may be accomplished in different ways depending on the type of fish which is being handled. Brailing salmon, pumping small fish such as herring, draining the fish and sea water through "port holes" or gates in the side of tanks, and individual handling, as with halibut, may be employed. A special study could be made of possible unloading devices which might be more rapid and convenient than those suggested above.

Effect of storage in refrigerated sea water on fish

Several years ago, when it became evident that the technique of transporting fish in refrigerated sea water offered distinct commercial possibilities, a study of the various effects of this mode of storage was initiated at our Station. It has been known for years that fish stored in ice usually lose weight. However, it was shown some years ago at our station that in fillets immersed in fresh water there was a transient gain in weight, but that this was subsequently lost after 1 or 2 days storage at 0° C. (10). A few years ago Cutting (11) demonstrated that with cod iced on trawlers, the fish in the upper layers usually lose little weight and may gain weight slightly. On the other hand, fish which are in the bottom layers of ice lose weight markedly. Thus, a 17 % loss in weight was recorded for fish stored in bottom layers of ice for 17 days in exceptional cases. These losses of weight do not occur with fish stored in refrigerated sea water. There is invariably some increase in weight, which is not unduly great with many varieties of fish. The increase varies with time of storage, size and fattiness of fish and whether or not they are eviscerated. With sockeye and small coho salmon increases of between 1.2 and 3.7 % were observed after 6 days storage (12) and with herring 3.5 % after a week's storage (13).

With halibut, no results concerning weight changes are available as yet as it has not been possible to weigh the fish accurately due to the violent motion of the comparatively small vessels when at sea. It is only with quite thin fish such as small "soles" (flat fish) that the weight increases are very high and may attain 18—20 %. Some moderate measure of success has been attained in experimental measures which have been taken to prevent these increases but a really satisfactory solution to the problem remains to be found (13), and at present it is inadvisable to keep soles for long in this storage medium. It would seem advisable to determine the effect of this method of storage on weight of fish in a given fishery before deciding to apply it. Early studies indicate that the method could be very satisfactory for Atlantic cod (2).

Changes in the inorganic ions in the flesh of fish stored in refrigerated sea water have been studied in considerable detail (13, 14). In sea water ordinary salt is the predominating crystalloid, and hence the concentration of sodium ions considerably higher than that of potassium ions. In sea fish flesh the reserve is true, for there are about 400 mg. per 100 g. of potassium ions and 50 of sodium ions, though these figures differ considerably with different fish and with their physiological condition. In flesh of fish stored in refrigerated sea water, after an initial lag period which appears to be related to rigor mortis, there is a gradual rise in the sodium ion concentration and drop in that of potassium ions. As with weight increase, the change will vary with the treatment of the fish, their size and fattiness. In general the sodium ion increase, when calculated as ordinary salt, is unlikely to exceed 1.5 %, and is usually between about 0.5 and 1.5 %. With salmon intended for canning this presents no problem, and so far no trouble has been experienced with halibut.

Studies have shown that protein losses, with salmon at least, are somewhat less in fish stored in refrigerated sea water than with those stored in ice (12). Since it has been found that three to four times as much fish as sea water can be stored in this medium, it would seem to offer significant possibilities as a practical medium for holding chilled fish.

Other physical methods of preservation

About two decades ago it was found that exposure to ultraviolet light (15, 16), even if it was continued for many hours, had an almost negligible effect on keeping quality of fillets. Since 1947, "radiation sterilization" of fish has been studied extensively, and an excellent review of the whole subject appeared a few years ago (17). Briefly, it appears that complete sterilization by penetrating electromagnetic radiations such as are obtained by use of high voltage electron accelerators or atomic fission products invariably occasions marked undesirable flavour alterations in fish. Some possibility seems to be offered by use of partially sterilizing (pasteurizing) doses alone, or in combination with antibiotics. Use of added free radical acceptors, or radiation treatment in vacuo or when the fish is frozen tends to minimize undesirable alterations. However, presently at least it cannot be said that there is any immediate hope of using this general method for retarding bacterial spoilage of fish. It is obvious that any treatment of fish should, if at all possible, be carried out on the fishing grounds in order that spoilage bacteria may be checked before they commence multiplication. Radiation sterilization on fishing vessels in the present rather technologically backward stage of this industry would not appear at all feasible. Recent work with oysters indicates some of the difficulties which may be experienced in development of off flavours and odours in treated sea foods (18).

Chemical preservation

The earliest chemical preservatives for fish were salt and wood smoke, and these two are still employed. The action of salt is probably largely due to dehydration and osmotic effects on bacteria involved in spoilage. The early literature on use of chemicals to retard fish spoilage was reviewed by the speaker in 1940 (19) and again in 1954 (20), and has been recently discussed in detail by Tomiyasu and Zenitani (21). The chemical agents which were used from time to time in different countries up to 1940, sometimes with and sometimes without government approval, were benzoic acid and certain of its esters, boric acid, carbon dioxide, hexamethylene tetramine, and hydrogen peroxide. None of these substances proved of outstanding value but some were moderately useful. In 1939 studies at our Station showed, for the first time, that potassium nitrite exerted a pronounced bacterio-static effect in fish muscle (22, 23). Subsequent research, most of which was carried out with sodium nitrite, verified these initial findings and a number of papers and articles were published relating to the mode of preservative action of nitrites on fish, their effect of pH (muscle acidity), on their activity, and their influence on the colour of salmon (19, 20). It was found that sodium nitrite, in concentrations ten times greater than the 200 parts per million (ppm) level which gave good preservative results with fish, was not apparently toxic when fed to rats and to cats (24).

During the last war sodium nitrite was used very extensively in the Canadian Maritime Provinces for fillet preservation and its use was permitted under the Canadian Food and Drugs Act. It is, in fact, still among the list of permitted food preservatives in Canada (25). During recent years the Norwegians have made extensive practical use of sodium nitrite in preservation of fish meals and have verified and extended our work

oncerning its non-toxicity when properly applied (26). Successful results in use of odium nitrite for fish preservation have been obtained in Europe (27, 28).

)uring our early investigations with sodium nitrite it became apparent that there were ertain objections to its use. Thus it is definitely a pro-oxidant and accelerates develop- ent of oxidative rancidity with frozen fish, though, strangely enough, it exerts a ild antioxidant activity with chilled non-frozen fish (29, 30). Nitrites combine with ny blood pigments which are present in fish muscles causing a pink colour which is ue to formation of nitrosomyoglobin. For these reasons, and because nitrite salts do ot exert a marked bacteriostatic effect unless the fish flesh has a faintly acid reaction . g. below pH 6.5), a more effective substance was sought. Early in 1942 we commenced n active "screening" program to ascertain the possible value of various chemical ubstances for preventing decomposition of fish by bacteria or other microorganisms. 'he British discovery of penicillin and of its importance in therapy of certain human liseases by Fleming and by Florey and others stimulated us to include antibiotics in ur investigations. At this time penicillin was not available to us so we made and tested . mould metabolic product, namely penicillic acid, which had been discovered earlier n Raistrick's laboratory. This proved to have a negligible preservative action for fish. n fact, none of a series of compounds tested at this time, including ethylene and ropylene oxides and chloramines, offered any promise for various reasons (31). When enicillin, streptomycin and certain "sulpha drugs" became available, we studied hese. Thus in a number of different experiments sulphanilamide, sulphathiazole, enicillin, streptomycin, certain quaternary ammonium or similar compounds and an cridine dye were incorporated in ice used for icing fish or were used in solutions in vhich fillets were dipped (32, 33, 34). None of these compounds, with the exception f the sulpha drugs, proved as effective as $NaNO_2$ in the concentrations in which they vere used. In fact, none of the substances studied by us prior to 1950 offered any greater promise in retarding fish spoilage than did sodium nitrite.

Early in 1950 we commenced tests with a number of new antibiotics which were kindly made available to us by different pharmaceutical firms, and as a result made an bservation which was destined to have far-reaching repercussions in the field of food reservation. It was found that Aureomycin (chlortetracycline, or CTC), Terramycin oxytetracycline or OTC) and chloromycetin all were extremely effective in retarding acterial spoilage of fresh fish (35). Two years later we published in some detail the esults of extended experiments in which were recorded the results of tests in which bout fifteen different antibiotics had been studied for their effectiveness in delaying acterial spoilage of fish and meats at temperatures between 0° and 20° C. (36). Briefly, he results showed that CTC was the most effective antibiotic for flesh food preservation, hat it was more effective than OTC, and considerably more effective than chlor- mycetin. The effectiveness of CTC was outstanding, for concentrations as low as 0.5 nd 1.0 ppm. inhibited bacterial spoilage of ground fish flesh very strongly.

From 1952 to the present time we have continued and expanded our work on use of ntibiotics in fish preservation; and other laboratories, stimulated by our success with ish and beef, have initiated research concerning the preservation of chicken, turkey, eef, pork and other flesh foods. In view of our early findings that CTC was the most ffective antibiotic for fish preservation, all our research for several years was carried ut with this antibiotic. The results of these investigations have been published and eviewed from time to time (37, 38, 39, 40) so only the significant findings will be mention- d. In our initial work with ices containing CTC, machines which prepared flake ice were sed since with this method of ice manufacture no problem of distribution of the ntibiotic occurred. In exploratory experiments ices containing 1, 2 and 4 ppm. of CTC

were made, and when used with well-iced lingcod, all three ices appeared to be about equally effective in delaying bacterial spoilage. As a result of these successful tests, one large British Columbia company suggested to us that we experiment with a small plant producing about four tons of flake ice daily to ascertain whether we could improve quality of salmon. The ice was prepared to contain a calculated 1.25 ppm. of CTC, and microbiological assays carried out from time to time showed an average of 1.07 ppm. in ice removed from fish boxes on arrival in Vancouver. Bacteriological and organoleptic examinations indicated that, with well-iced coho salmon *(Oncorhynchus kisutch)* and red spring salmon *(O. tschawytsch)*, the CTC ice exerted a very important improvement in keeping quality. Conservative estimates indicated at least a 5-day increase in keeping quality in comparison with fish stored in ordinary ice. Last summer it was suggested that we conduct tests in the "groundfish" fishery. In this fishery large vessels use otter trawls to catch different species of "soles" (flatfish), grey cod *(Gadus macrocephalus)* and "sea bass" *(Sebastodes* species). These fish do not command nearly so high a price as do halibut and salmon, and are therefore usually not iced very carefully, and are not eviscerated at sea. Consequently, there is a serious bacterial spoilage problem. Block ice containing 2.5 ppm. of CTC was prepared, using carboxymethylcellulose in a method to be referred to below, and this ice was crushed and used in icing experiments in comparison with ordinary crushed block ice. The level of CTC was higher than that used in previous trials in view of the usual poor icing procedures and because in one instance permission to use 5 ppm. in ice had received official government permission (see below). In this experiment adequate sampling of the fish proved impossible with our limited facilities because the vessels landed 50 000 to 80 000 lb of fish. Assays of the limited samples obtained, together with the reaction of the vessel captains, indicated that there was a marked overall improvement in quality of the fish, and that fish iced with the CTC ice was not discarded for reduction to fish meal as sometimes occurs with fish iced with ordinary ice. In addition to our own trials large-scale tests were carried out by a Northern British Columbia firm with ice containing 2.5 ppm. of CTC in the Alaska area halibut fishery. Professional halibut graders and officials who are familiar with halibut reported that the fish landed were in unusually good condition in comparison with fish normally landed from this area.

In addition to our own trials reported above there are available published reports of the effectiveness of antibiotics in fish preservation from several other well-known laboratories. A few years ago a California laboratory reported that immersion of fillets in 2-ppm. solutions of CTC and OTC caused marked reduction in their susceptibility to bacterial spoilage, the former antibiotic being somewhat more effective than the latter (41). A more recent report from the same laboratory (42) showed that a two-minute immersion in 5-or 25-ppm. solutions of CTC, OTC or tetracycline (TC) caused a marked improvement in keeping quality of fillets stored at 6° C. and that CTC was the most effective of 14 antibiotics investigated.

During the past two years Shewan of the Torry Research Station has been responsible for conducting a number of trials concerning the effectiveness of CTC ice in the British cod fisheries. In initial work he found that this ice extended the keeping quality of cod about seven days (43). Last year he reported that ice containing 5 ppm. of CTC was much more effective than certain other chemical compounds tested, and stated that with white fish such as cod and haddock stored in CTC ice, "the point of inedibility" was reached some seven to ten days after it was reached in the "controls" (44). In Japan Tomiyama and his collaborators have conducted very extensive trials with CTC in fish preservation. It is impossible to cover in any detail the results of their experiments. Briefly, they found that very marked improvements in keeping quality of round herring

ıd sardines, and of mackerel, occurred when these fish were, in various experiments, ·ld in refrigerated sea water containing CTC, in ice containing 5 ppm. of the antibiotic, - were dipped for an hour or two in 10—40-ppm. CTC solutions (45, 46, 47, 48). Extensi- e studies in the United States have verified and extended our early findings regarding ıe value of CTC ice in extending the storage life of Atlantic fish (49).

bout a year ago the Fourth Annual Symposium on Antibiotics held in Washington, atured for the first time a number of papers concerning the use of antibiotics in food reservation, and a discussion panel on the subject (50). One of the papers presented ·viewed work which was carried out with 19 antibiotics in experimental preservation f fish. In this work CTC proved more effective than OTC and TC (51). In other papers ı which rather high concentrations of three tetracycline antibiotics (5-to 20-ppm.) ere compared for their effectiveness in preventing spoilage of salmon species and of ıle stored in chilled salt solutions it was reported that all were about equally effective 52, 53). Our recent work, which was reported last May at the Annual Meeting of the nstitute of Food Technologists in Pittsburgh (54) has verified previous findings that TC is consistently more effective than OTC or TC when used in fillet-dipping or fish- ·ing procedures, and also appears to be more effective when used with fish held in ·frigerated salt solutions. In refrigerated sea water the comparatively anaerobic onditions which prevail cause a different type of spoilage from that found with iced sh, and a detailed study of this rather unusual spoilage pattern is in progress. The accessful applications of CTC to Atlantic rosefish was also reported (55) at this meeting.

n general, though CTC has proven of considerable value in retarding spoilage of shell- sh such as oysters (56, 57), cooked crab meat (36) and shrimp (41, 58), the concen- rations required for successful preservation have been considerably higher than those hich have been successful with fish. So far no adequate explanation of this fact has een advanced. It is doubtful if the pH of the tissues is a factor since oyster meats sually possess a slightly acid reaction, while with shrimp and crab meat the reverse s true. Preservation of menhaden for reduction has been successfully accomplished by se of a CTC spray (59).

he problem of preservation of whales is difficult since the carcasses of these warm- looded mammals undergo extensive anaerobic bacterial spoilage if they are not- rocessed in 16—24 hours after capture. During the past two whaling seasons we have arried out trials in a local shore whaling operation in which 50 or 100 grams of pure TC were dissolved in about 20 gallons of sea water and injected into the visceral avities of the animals. The results of initial tests were so promising (60) that last year 5 whales were treated. Bacteriological examinations of tissues taken from seven egions in each whale indicated a considerable overall improvement in quality, and the ork is being extended this year. It is planned to conduct more extensive trials in hich the antibiotic will be used in the harpoon head as well as introduced by the njection method. It is hoped that the results of these tests will eventually be published.

Antibiotic residues in fish flesh and public health aspects

t has recently been pointed out (61) that, from the point of view of their use as food reservatives, antibiotics should be considered in common with other potential food dditives. There is, indeed, no hard and fast dividing line between antibiotics and hemical compounds. Thus, sodium nitrite is formed by microorganisms and could be onsidered an antibiotic, while certain antibiotics have been prepared by total chemical ynthesis. It is unfortunate that the term antibiotic has been loosely associated with nedical usage, and has thus been automatically linked with development of hyper-

sensitive reactions, development of resistance by individuals so that they can no longer rely on antibiotic therapy, or with resistance of parasitic bacteria so that they can no longer be controlled by antibiotics. These dangers, real or imaginary, are the subjects of much confused thought not only by laymen but by people whose training should enable them to evaluate each situation on its own merits. Problems in the use of CTC, or other antibiotics, in fish must be regarded in the same light as are those of other food additives.

With poultry there is no real problem with residues of CTC since normal cooking procedures change the antibiotic entirely (62, 63) to isochlortetracycline (64). On the other hand, the comparatively light cooking procedures which are usually advocated for fish may result in very small amounts of CTC remaining in the product after cooking. It was shown in our laboratory two years ago that about 95 % of added CTC is destroyed by heating fish flesh 5 minutes at 99° C. (65). More recently (66) we have studied the effect of certain normal fish-cooking procedures on the amounts of CTC which remain in fish which had been iced with ices containing between 2- and 4-ppm. of CTC in the laboratory, or on fishing vessels. In the uncooked fish the highest residue found was 0.6 ppm. in an unskinned fillet. Residues in the cooked flesh varied with the method of cooking and with the area sampled, amounts of from 0 to 0.3 ppm being found. It was observed that, since the dark skin of fish is rarely consumed, and antibiotic residues are highest in this region, it is highly unlikely that consumers of CTC-treated fish will receive over 0.1 ppm. of the antibiotic. CTC residues in whole eviscerated fish stored in refrigerated sea water, or dipped in CTC solutions, are inclined to be higher than those found with iced fish, but the values are very variable depending on the size and variety of fish and the concentrations of CTC used. The values reported for uncooked fish by United States investigators (49) in general compare favourably with our results recorded above. Recently Tomiyama et al (67) have shown that fish held from about one to two days at 4° C. in sea water containing 10 ppm. of CTC contain from 0 to 2.8 ppm., but that the antibiotic is, after this comparatively short storage period, in the skin and gills of the fish and not in the flesh or viscera. With the experimental technique employed these investigators found that it would require 26 minutes at 93° C. to inactivate 90 % of CTC in fish muscle. Shewan (68) has recently observed that, "Indeed, in the skinned, minced raw muscle from fish stored 28 days in ice containing 5 ppm. Aureomycin it has been difficult to detect any Aureomycin at all." On the other hand, he found quite high concentrations (3.5—11.1 ppm.) in the skin. It would appear from the results obtained in these four widely-separated laboratories that typical applications of CTC to fish for preservative purposes will usually result in small but detectable residues in the raw uncooked fish. However, these residues are concentrated mainly in the skin, or gills, of the fish, and are largely or entirely inactivated by heating. With concentrations of CTC of the order mentioned above it would be necessary to consume about one ton of raw fish or ten tons of cooked fish to ingest one normal daily therapeutic oral dose (1 gram) of the antibiotic.

Warnings regarding the potential danger to public health in use of antibiotics are continually being raised (69, 70, 71) but in no instance so far have these had any sound experimental or factual bases. It has been pointed out that some of the questions which have been raised can only be answered by experience and not by experiment (69). It may be recalled at this time that the inclusion of certain medically valuable antibiotics in animal feeds to promote growth and well-being in certain farm animals, which has now become a standard procedure, was considered by many as a potentially dangerous procedure. After about seven years of widespread application the effects on the animals appear to have been uniformly favourable (72), and we are told that

bout one quarter of the antibiotics now produced in the United States are used for is purpose. Another interesting and important point is that, though research in the nited States has shown that about 5 % of market milk samples contain significant nounts of penicillin, which arise as a result of use of this antibiotic in treatment of ws for mastitis, in no case has any instance of hypersensitive reaction in humans en traced to ingestion of such milk. Penicillin is one antibiotic to which humans adily develop hypersensitivity.

he general harmlessness of CTC when administered orally is well known, and this hole subject has been ably reviewed recently (73). Premature infants, infants and ildren have been fed daily oral doses of CTC ranging from 20 to 50 mg. in some stances for 35 months, without any harmful effects. Though nausea may arise in dult patients treated with therapeutic doses of CTC, the available literature indicates at at comparatively low levels the antibiotic is tolerated well with no cumulative xic effects, even after many months. In geriatric patients long-term administration f CTC has never been followed by lasting toxic manifestations. The extraordinary bility of rats to tolerate CTC is brought out by an experiment in which these animals ere fed a ration containing 1 % of the antibiotic with no adverse effects, when ompared with appropriate control animals. Experimental data indicate that isochlor-tracycline, the bacteriostatically inert compound which arises from CTC when it is eated as in cooking of flesh foods, is also completely non-toxic. In summary, it would ppear that there is much more information presently available regarding the lack of xcity of CTC than has been accumulated for certain other food additives which are sed in comparatively much higher concentrations.

We have recently shown that small amounts of CTC in fish flesh actually tend to uppress development of vegetative cells of *Clostridium botulinum* (type E) and of an nterotoxigenic (food poisoning) strain of *Staphylococcus aureus* (39, 40). The effect n Salmonella organisms has not as yet been determined.

The only potential danger in use of antibiotics such as CTC in fish, or other flesh oods, would appear to lie in misuse. Application to fish of poor bacteriological quality s useless; only strictly fresh fish should be treated. Bacteria can develop slow resistance o CTC, for, as an example, we have developed, over a period of many months, a marine acterium which grows well in media containing 1000 ppm. of the antibiotic (39). ortunately, if fishing vessels are kept clean, the danger of resistant bacteria developing n fish which are iced with CTC ice is extremely remote. Each fish which is brought board has a fresh bacterial flora which has never had opportunity to develop CTC esistance. On the other hand, fillet-dipping procedures could be a source of trouble f the tanks and equipment used are not properly cleaned and sterilized. As with any ther food process, unless the procedure is properly carried out under prescribed onditions, failure or complications may result.

t this time the following permissions have been granted in North America for use of tracycline antibiotics an flesh foods. In Canada, on September 26, 1956, the Food nd Drugs Act and Regulations were amended as follows: "No person shall sell fish hat has in or upon it chlortetracycline if (a) the amount thereof exceeds 5 parts per illion; (b) its presence is not declared by name on the main panel of the label." More ecently the use of oxytetracycline has been permitted under similar conditions. In oth the United States and Canada the inclusion of not more than 7 ppm. of CTC or TC is permitted in uncooked poultry. Since much of Canada's fish is presently ex-orted to the United States the application of antibiotic treatment to fish is presently mited to products which are consumed domestically. Shellfish such as oysters have

not been included in the above regulations because they may be consumed uncooked and properly treated oysters will contain significant antibiotic residues (56, 57). At this stage serious consideration should be given to the desirability of permitting inclusion of small residues of tetracycline antibiotics in foods intended for human consumption. Available information does not indicate that there could be any cumulative or other toxic reactions as a result of such inclusion, and development of hypersensitive reactions to small oral doses of tetracycline antibiotics does not appear to occur. It is even possible that, as has occurred in feeding antibiotics to young farm animals, the results of such additions could be beneficial.

Miscellaneous considerations

Certain difficulties have been experienced in our work on fish preservation by use of CTC. The problem of obtaining uniform distribution of tetracycline antibiotics in block ice arose several years ago, and this was solved independently in two laboratories by addition of the collodial gelling agents carboxymethylcellulose and carrageen (74, 75, 76). Low concentrations of these agents of the order of 0.01 % are adequate for occasioning comparatively even distribution of tetracycline antibiotics. Another problem which has arisen in our work and which has not yet been solved satisfactorily is that of the presence of substances in muscles of certain fish which occasion slightly positive CTC microbiological assays in the absence of added antibiotic. These substances seem to be present in most of the fish with which we have worked, and it is for this reason that in work on antibiotic residues in fish we have normally used grey cod, the flesh of which rarely gives these "fictitious assays". These substances appear to be soluble in certain organic solvents and may be lipoidal in nature (39). Flesh extracts obtained by the acid-acetone method or by extracting it with water at pH 4.5—4.6 are both capable of containing these interfering materials. Since these materials may cause false assays which are equivalent to about 0.25 ppm of CTC, it can be appreciated that it may be impossible to distinguish readily traces of the antibiotic in flesh of some treated species of fish unless tedious extraction and chromatographic techniques are applied (39).

In conclusion, it must be emphasized that we are continually searching for better preservatives to retard bacterial spoilage of fish, and for preservatives which retard or prevent yeast and mould growth on this commodity. Thus, sorbic acid has proven valuable for the last-named purpose (77), and a number of new antifungal antibiotics are now under investigation.

Zusammenfassung

Der Vortrag gibt eine zusammenfassende Darstellung des Frischfischverderbs und seiner Kontrolle mit verschiedenen Methoden. Die Beobachtung, daß der bakterielle Verderb bei 2,5° C etwa doppelt so schnell wie bei —1° C verläuft, bahnte den Weg für alle Arbeiten, praktische Methoden ausfindig zu machen, den Fisch bei —1° C vom Fang bis zur Verarbeitung zu lagern. Versuche zur Temperatursenkung durch Anwendung spezieller Eissorten haben gezeigt, daß Eissorten mit einem Gehalt von 1 bis 3 % Seesalz etwas besser als Frischwassereis konservieren. Guten Erfolg versprechen Versuche mit der Fischaufbewahrung in gefrorenem Seewasser bei —1° C. Die Gefahr der galvanischen Wirkung und der damit verbundenen Korrosion kann dadurch beseitigt werden, daß man Tanks, Kühlschlangen usw. aus korrosionsbeständigem Aluminium herstellt oder mit Kunstharzanstrichen versieht. Rascheres Gefrieren läßt sich durch besondere Wärmeaustauscheinrichtungen erzielen.

Bei einer Lagerung in gefrorenem Seewasser kann es zu Gewichtsveränderungen bei den Fischen kommen, der Gewichtszuwachs hängt von Lagerungsdauer, Größe und Fettgehalt des Fisches

b. Bei Schollen, Seezungen kann der Gewichtsanstieg bis 20 % erreichen, während er bei anderen ischen nur gering ist. Trotz aller Bemühungen ist eine befriedigende Lösung dieses Problems och nicht erzielt. Veränderungen in der anorganischen Ionenzusammensetzung des Fischfleisches ährend der Lagerung in gekühltem Seewasser — allmählicher Anstieg der Natrium- und Abfall er Kaliumionenkonzentration —, variieren in Abhängigkeit von dem Gewichtsanstieg und Fettehalt und hängen auch von der Behandlung und Größe des Fisches ab. Der Natriumionenanstieg egt zwischen 0,5 und 1,5 %. Eiweißverluste sind weniger ausgeprägt als bei in Eis gelagerten ischen.

terilisierung durch harte elektromagnetische Strahlung (Hochspannungs-Elektronenschleuniger oder Atomzerfallprodukte) ruft unerwünschte Geschmacksveränderungen im Fisch ervor.

hemische Konservierung mit Natriumnitrit hat guten Erfolg und zeigt bei richtiger Anwendung keine toxische Wirkung. Viele dem Eis beigelegte bakterizide Stoffe, wie Penicillin, treptomycin, Sulfanilamid, Sulfathiazol und eine Reihe quarternärer Ammoniumbasen, zeigten it Ausnahme der Sulfadrogen keine so gute Wirkung wie das Natriumnitrit.

n Versuchen mit neueren Antibiotika wurde festgestellt, daß Aureomycin (Chlorotetracyclin CTC) die beste Wirkung hat. Schon 0,5 bis 1 mg/kg hemmt den bakteriellen Verderb. In raktischen Versuchen auf Fischdampfern zeigte sich, daß 2 min Eintauchen in 5 bis 25 mg/kg ösungen von CTC eine deutliche Verbesserung in der Qualitätserhaltung von bei 6° gelagerten ilets verursacht. Es konnte nachgewiesen werden, daß die übliche CTC-Anwendung für Fischonservierungszwecke gewöhnlich zu kleinen aber nachweisbaren Überresten im rohen, ungekochten Fisch führt. Diese Reste sind vorwiegend in Haut und Kiemen abgereichert, werden aber weitgehend oder vollständig durch Erhitzen inaktiviert.

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EFFECTS OF DDT SPRAYING IN NEW BRUNSWICK ON FUTURE RUNS OF ADULT SALMON

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Seining with electro-fishing at a young salmon sampling area in the Northwest Miramichi. Each season a total of about 1,000 such collecting operations are made on ten sampling areas in this one tributary, to give a reliable estimate of the number of young of various sizes. The fish are released uninjured after examination.

A RECENT OUTBREAK of spruce budworms has threatened the woodlands of northern New Brunswick, and has resulted in the use of a powerful control method—aerial spraying of DDT insecticide. Some of our best Atlantic salmon producing streams run through these valuable wooded areas. Unfortunately, young salmon stock may suffer extensively from the insecticide which enters their water environment. A predicted depletion of adult salmon has been given some publicity, but is seems desirable to discuss various aspects of the DDT and fish relationship to improve public understanding.

Since the first use of DDT to control harmful insect pests in forested areas, it has been known that it could have quite harmful effects on other organisms, particularly small fish. Laboratory tests in 1944-45 at Algonquin Park, Ontario, showed that small eastern brook trout and other species of fish were killed when minute traces of DDT-in-oil solution were added to the water in which they were held. Observations over the past twenty years in streams of many parts of North America during and after application of DDT-in-oil insecticide, have led to conflicting opinions about the effects on fishes. In many cases no serious

direct effects have been observed on members of the trout (char) and salmon family. For example, careful observations in 1956 in Montana indicated that caged rainbow and eastern brook trout, ranging in length from three to eleven inches, escaped apparent injury when DDT insecticide was sprayed at the rate of one pound per acre. On the other hand, observations since June, 1954, by staff of the Fisheries Research Board of Canada and the Conservation and Development Service of the Federal Department of Fisheries, showed that a very high proportion of young Atlantic salmon were killed when woodland in the watershed of the Miramichi River was sprayed with DDT-in-oil insecticide at the rate of one-half pound of DDT per acre. Similarly, spraying of woodland in northern Vancouver Island, B.C., in June, 1957, with DDT-in-oil against black-headed budworms was followed by the death of many coho salmon fry and young steelhead trout. In the British Columbia operation various precautions were taken in the hope of avoiding undue pollution of the streams with insecticide, based on our experience with Atlantic salmon in northern New Brunswick.

These points have been mentioned to indicate the complexity of the problem. It is now apparent that the effects of DDT spraying on fishes may be quite variable among different areas. The geographical and geological features of the area, the kinds of fish occurring there, and the distribution of various size groups of each species, apparently have a significant influence on the extent of damage to fish populations.

Why is aerial spraying of DDT insecticide used for the control of budworms and other forest insect pests when it may kill many valuable fish? This obvious question should be considered early in a discussion of this kind, to prevent unjustified prejudice against current forestry practice among those who may be more interested in fish than in trees. Much of this is outside the writer's field, but an effort will be made to summarize present views of agricultural, forestry and fishery specialists as expressed during recent discussions.

Projects to control forest insect pests in Canada by direct application of chemical insecticides have been aimed exclusively at reduction of harmful insect populations in specific places and at specified times. No attempt is made to eradicate forest insects which are normally present in moderate numbers. Large-scale chemical control methods have become economically and operationally possible only within the past few years. Aerial spraying of insecticide has been adopted as the only known effective method of controlling outbreaks of forest insects in large, relatively inaccessible forest areas. It is expected that further improvements in techniques will develop through research, and that chemical control will continue to be used against an increasing number of pests. In northern New Brunswick where large, remote areas of woodland, containing much of the highly susceptible balsam fir, have been subjected recently to an outbreak of the spruce budworm, DDT spraying was chosen as the only way to provide effective protection of existing reserves of valuable forest. Other known forest management techniques, involving biological control, rotational cutting or other silvicultural methods, would be very long-term operations and ineffective in the present emergency.

From the fisheries standpoint it is reasonable to assume that without adequate forest cover to control water run-off into salmon streams, there would be poor prospects for rearing needed supplies of young fish. Par-

ticularly in the smaller tributaries, the adjacent forest cover helps to maintain moderate water temperatures by providing favourably shaded conditions. When chemical control of the recent budworm infestation was first considered, the Federal Department of Fisheries realized that much of the forested area adjacent to salmon streams was threatened. Introduction into fish-populated waters of chemicals or other deleterious substances is prohibited by the Fisheries Act. In these circumstances, however, it was necessary to attempt to reconcile the interests of the fisheries and forestry resources. It was agreed that DDT spraying should be undertaken but that every effort should be made to obtain information on its effects on the fish populations. This would indicate the need for development of improved techniques of chemical control of insect pests, and of new fish-cultural practices to overcome fish deficiencies caused by the insecticides.

By 1954 the Fisheries Research Board of Canada was in an unique position to learn the effects of DDT spraying on Atlantic salmon, because woodland in the Miramichi area, N.B., was included, for the first time, in the spray plans. Since 1950 the Board had been studying Miramichi salmon and had assembled a good record of the abundance of young salmon in two tributaries. This information was obtained by systematic seining operations using electro-fishing techniques on a series of sample areas. The data provided estimates of the numbers of young salmon of each of the three size groups—fry, small parr and large parr—living in unit areas of the river prior to spraying. One of the tributaries was the Northwest Miramichi, whose watershed was thoroughly sprayed in June, 1954 from the headwaters for a distance of forty miles downstream. The regular autumn seining that year showed that all size groups of young salmon had been seriously affected. The smallest, hatched that spring, were practically eliminated in the sprayed area. Effects on the larger sizes were less severe, but the population of small parr was cut to about one-sixth the pre-spray level, and the population of large parr to about two-thirds the normal level of the pre-spray period. Besides these observations on "wild" fish in the stream, studies were made on young salmon, $2\frac{1}{2}$ to $4\frac{1}{2}$ inches long, that had been seined in the river and then placed in cages both inside and outside the area to be sprayed. Those in sprayed tributaries suffered high mortality (up to 84%) within two weeks of the start of spraying upstream, while no deaths occurred in the outside controls in this period. Within two days of upstream spraying, large numbers of young salmon, as well as other species of fish, were seen dead and dying along the river banks. This has been considered to be largely a direct effect of the insecticide on the fish, as had been observed elsewhere in laboratory experiments for species other than salmon. Also observed was the quick disappearance in sprayed areas of most of the stream insects on which young salmon feed. These include the caddisflies, stoneflies, mayflies, blackflies and midges, whose larval stages are spent under water on the stream bottom. From 1955 to 1957 other parts of the Miramichi system have been sprayed, and some areas have now been sprayed from one to three times. Observations on young salmon and their food have been continued on the Northwest Miramichi and other tributary streams.

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Since 1955 much more detailed work has been undertaken on some aspects of the problem, particularly on the effects of DDT on survival of stream insects. The smaller kinds of these

insects, for example the midges and blackflies, which are very suitable food for salmon fry, have shown marked ability to become re-established quickly. Many of the larger forms, such as caddisflies, have not reappeared and efforts were started in 1957 to transplant them from unsprayed areas. More details of these investigations are given in reports published each spring in the Department of Fisheries *Trade News*. Reprints of the reports for 1955 and 1956 are available upon request from the Biological Station, St. Andrews, N.B. In 1957, studies on young salmon were extended to the Tobique River, N.B., and effects of DDT were observed similar to those in the Miramichi area.

No harmful direct effects of the DDT sprayings on adult salmon entering the Miramichi River have been observed. In 1954 a normal run of adults ascended the Northwest Miramichi, and in 1955 an unusually abundant population of fry resulted here from the spawning in the fall of 1954. These have survived reasonably well in the upper areas that have not been re-sprayed since 1954. In lower areas that have been sprayed again, this year-class has been lowered to an insignificant level. The smolt production of this tributary is now at a low ebb and can be expected to remain low for several years.

What effects will such decreases in production of young salmon have on future stocks of adults, which depend entirely on the smolt production of freshwater areas? Facts about salmon movements and utilization which are important in this connection are: (1) Atlantic salmon usually return as adults to the freshwater tributaries in which they were produced; and (2) salmon produced in a particular river, say the Miramichi, may be taken as adults in commercial sea fisheries over a wide area of the coast, as far away as Newfoundland and the Labrador coast.

In January, 1958 an effort was made by staff of the Fisheries Research Board of Canada, Biological Station, St. Andrews, N.B., to forecast the effects of past sprayings with DDT on future stocks of adult salmon that will be available to commercial fishermen and anglers. The problem of forecasting is a difficult one for several reasons. These include: (1) The life cycle of Atlantic salmon is quite long. The period often needed to give large salmon weighing from eight to twelve pounds is six years with the last two years spent in the sea beyond the scope of investigation. (2) Catches by commercial fishermen and anglers are the only available index of abundance of adult salmon for many of the sprayed areas. Since catches are affected greatly by weather and water conditions, skill of the fishermen and other factors, it is difficult to estimate the extent to which variations in catches reflect variations in actual abundance of fish. (3) In the Miramichi and Tobique river systems, systematic seinings have been undertaken to give estimates of the abundance of various stages of young salmon, thus the actual effects of spraying on the different year-classes or generations of young are known. Such quantitative information is lacking, however, for other parts of the area affected by DDT. The effects of spraying, in a large fraction of the river areas that have been sprayed, can only be estimated on the basis of qualitative observations of various kinds, combined with quantitative data for similar sections of the Miramichi and Tobique watersheds.

Dr. P. F. Elson of the St. Andrews staff has made the calculations needed to predict the effects of DDT sprayings in the Miramichi, Restigouche, and upper St. John rivers. This work included the following operations: (1) Measurement on maps of the total areas of each watershed which have

been available for spawning of adult salmon and the rearing of young. (2) Calculation of the proportion of each of the above areas which was affected by DDT in each year, taking into account the position of the sprayed area on the stream. (3) On the basis of quantitative data for the Northwest Miramichi, calculation of the percentage kill of each of the three size groups of young salmon in each year, within the other sprayed areas. A reasonable margin was allowed to avoid a possible over-emphasis of the severity of the effects of spraying. These calculations gave, for the different rivers, the percentage survival of fry through to large parr (which would become smolts and descend to the sea the following spring). (4) Conversion of these indices of large parr abundance to estimated numbers of adult grilse and large salmon that would return to the various fishing areas. Here it was necessary to estimate the proportions of each smolt run that would return as adult fish after one, two, or more years in the sea. These proportions were estimated from annual records obtained at special traps for counting upstream migrant fish on the Miramichi and Tobique Rivers, and from angling records for the Restigouche and main St. John River.

Because of the variable conditions which affect success of salmon fishing, particularly salmon angling, it is possible that a decrease might occur in actual stocks of adult salmon until they reach about one-half of the "normal" level, without any apparent difference in catch. Here "normal" simply means the total annual catch, whatever it was, before DDT spraying could have had any effect on it. To make these forecasts, graphs were prepared to show when the adult stocks of each river system would likely fall to the "50%-of-normal" and then to the "25%-of-normal" level.

No attempt will be made here to give details of these forecasts, but the main features for each of the three rivers may be summarized as follows. In the Miramichi area as a whole, the stock of adult salmon, including both grilse and larger salmon, is expected to decline to below the "50%-of-normal" level in 1960, and to the "25%-of-normal" level by 1961. In the Restigouche River, adult stocks are estimated to have fallen to the "50%-of-normal" level by 1957, although better angling was experienced there in 1957 than in 1956; they should recover somewhat in 1958 and 1959 but then decline again and reach the "25%-of-normal" level by 1961. In the upper St. John River, above Beechwood, a decline starting in 1955 will likely reach the "50%-of-normal" level by 1960, and "25%-of-normal" by 1961. It is important to realize that for two or three years yet, the serious effects of recent DDT spraying on young salmon survival in northern New Brunswick are unlikely to be apparent in the level of angling success enjoyed by the public in most areas.

Ever since the effects of DDT spraying on young Atlantic salmon became apparent in the Northwest Miramichi in 1954, serious consideration has been given to possibilities of overcoming the harmful effects by fish-cultural methods. Positive steps have already been taken by the Conservation and Development Service to produce increased quantities of large parr in hatcheries for release on rivers just before they will migrate to sea as smolts. Cooperative experiments by this Service and the Fisheries Research Board have shown that such operations are possible but there are still many unsolved problems. Owing to the high cost of hatchery rearing, it is doubted that artificial smolt production could ever approach the level normally resulting from natural reproduction, say two

million smolts, from the Miramichi river system. So far there are no data on the extent to which planted smolts survive to become adult fish in our waters. Nor is it known to what extent they will return as adults to the same rivers in which they are planted.

There are poor prospects for good catches of salmon over the next few years by commercial fishermen and anglers in areas affected by the recent sprayings. However, the actual effects of spraying on returns of adult fish to any particular tributary of a river system depend on the history of spraying in that area, and many factors like weather conditions at time of spraying. It is possible that many important angling stretches within the rivers mentioned above may have escaped serious injury, and that worth-while runs of adult salmon will continue to come to them. Since the commercial nets sometimes have a chance to catch salmon originating in many rivers, often far away, their catches may not be affected as seriously as anglers' catches in heavily sprayed areas of streams. Fortunately, the adult salmon that are available for capture in any year are not all of one age group, but are a mixture of several year-classes. This should allow some adult fish to be available continuously, even though individual year-classes of young salmon may have been practically eliminated. In this connection it is particularly unfortunate that the same areas of stream must be re-sprayed with DDT in closely succeeding years in order to preserve the trees.

In making these forecasts an effort has been made to analyse the situation objectively, using all the information that is now at our disposal. It would be unfair to withhold such information and run the risk of having the fishing groups or other agencies blamed a few years hence for a lack of salmon that may result largely from DDT spraying.

In conclusion, I should say that both Government and the forest industries are aware of this problem and anxious to find a solution. Forest Protection Limited, which does the spraying, and also the Forest Biology Division of the Canadian Department of Agriculture have facilitated our studies in every way possible. At present no suitable alternative insecticide is known that is less toxic to fish than DDT. Recently, however, an inter-departmental committee was formed in Ottawa to promote more intensive research on the problem and to ensure that spraying projects are not undertaken without giving full consideration to all the resources that may be affected.

LINGCOD MUSCLE PURINE NUCLEOSIDE PHOSPHORYLASE[1]

H. L. A. Tarr

Abstract

A purine nucleoside phosphorylase enzyme preparation, which catalyzed the general reaction ribose(deoxyribose)1-phosphate+purine⇄purine nucleoside (deoxynucleoside)+orthophosphate, was isolated from muscles of the lingcod (*Ophiodon elongatus*). The reaction was found to be about 85% in favor of nucleoside synthesis with 10 μM./ml. concentrations of reactants in the hypoxanthine–inosine system. With similar concentrations of reactants in the guanine–guanosine system the equilibrium was about 75% in favor of nucleoside synthesis, and with considerably higher concentrations of reactants in this system this percentage decreased markedly. Purified preparations were stable for over a year at −20° C., contained two major protein components (zone electrophoresis and ultracentrifuge data), were rather heat labile, and possessed a maximum activity corresponding to liberation of 32 μM. orthophosphate/mg. protein N hour. Evidence is given in support of the view that a single enzyme is responsible for the above reaction and that it possesses strict specificity for α-D-ribofuranose 1-phosphate and deoxyribose 1-phosphate. Of a large number of substituted purines investigated, only the following were active: hypoxanthine, xanthine, 6-mercaptopurine, 6-methylpurine, 8-azaguanine, guanine, adenine, and 2,6-diaminopurine. Methods are given for the preparation, in good yield, of analytically pure dicyclohexylammonium salts of ribose 1-phosphate and deoxyribose 1-phosphate.

Introduction

During an investigation of the riboside hydrolase enzymes of the muscles of Pacific lingcod (*Ophiodon elongatus*) (1) it was noticed that alcohol extracts of the tissues, when submitted to paper chromatography under appropriate conditions, contained small amounts of a substance with properties similar to those of ribose 1-phosphate. Subsequent experiments have shown that the muscles of this fish possess a purine nucleoside phosphorylase enzyme. Since initial brief communications concerning this enzyme were published (2, 3), it has been shown elsewhere that a guanosine phosphorylase is present in the skin of the roach (*Leucisus rutilus* Lin.) (4). No information has been given regarding the substrate specificity of this enzyme.

Materials

The following substances were obtained from the sources indicated: adenine, guanine hydrochloride, xanthine, hypoxanthine, guanosine, xanthosine, deoxyguanosine (Schwarz Laboratories, Mount Vernon, N.Y.); 2-aminopurine, 2-mercaptopurine, 2-oxypurine, 2-thioguanine, 6-methylpurine, 2-thio-6-oxy-8-azapurine, 4-amino-5-imidazolecarboxamide, 2,6,8-trioxypurine (uric acid), deoxyadenosine, crystalline 2-D-deoxyribose and its anilide (California Foundation for Biochemical Research, Los Angeles, California); purine, isoguanine-sulphate, 8-azaguanine, 8-azaxanthine, 8-chloroxanthine, uracil, ribose (Nutritional Biochemicals Corporation, Cleveland, Ohio); cytosine, 6-mercapto-

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Contribution from the Fisheries Research Board of Canada, Technological Station, Vancouver, B.C.

purine (Sigma Chemical Company, St. Louis, Missouri); 2,6-diaminopurine (General Biochemicals, Laboratory Park, Chagrin Falls, Ohio).

Synthetic α- and β-D-ribofuranose 1-phosphates (5, 6) and D-arabinose 1-phosphate (mixture of α and β isomers) were kindly supplied as their barium or dicyclohexylammonium salts by Drs. H. G. Khorana, G. M. Tener, and R. S. Wright of the British Columbia Research Council. The preparation of natural α-D-ribofuranose 1-phosphate (R1-P) and deoxyribose 1-phosphate (DR1-P) is described later. Before conducting enzyme experiments barium was removed from solutions of the barium salts by treatment with a little Dowex 50 resin in the ammonium cycle. For most experiments the dicyclohexylammonium salts were used. The Dowex 1×10 and Dowex 50×8 ion exchange resins employed were 200–400 mesh analytical grades obtained from the California Foundation for Biochemical Research. The XE 64 grade of the cation exchange resin Amberlite IRC-50 was purchased from Rohm and Haas, Philadelphia 5, Pennsylvania.

Methods and Preparations

Paper Electrophoresis

The technique employed was similar to that used in previous work (1) except that the concentration of stained protein was determined directly by means of a Photovolt densitometer.

Paper Chromatography

The following solvent systems were used with Whatman No. 1 paper: (*a*) 8 hours ascending development at 0° C. with *n*-butanol – glacial acetic acid – water (1:1:0.5, by volume) (1); (*b*) 15 hours descending development at room temperature (20–23 °C.) with *n*-propanol – 28% ammonium hydroxide – water (6:3:1, by volume) (7). Ribose and deoxyribose and their monophosphate esters were identified by means of the aniline hydrogen phthalate reagent of Partridge (8) containing 0.5 ml. of concentrated HCl per 100 ml. (1). The molybdic acid spraying reagent of Hanes and Isherwood (7) was employed for detection of orthophosphate (P_i).

In initial work the concentration of R1-P in enzyme mixtures was determined by quantitative paper chromatography using solvent (*a*) in a method similar to that used previously for determination of ribose (9). It was found that comparatively high concentrations of this ester were required (5 to 20 μg. applied in 5 μl. amounts; OD range 0.2 to 0.5 at 400 mμ).

Ribose was determined by the method of Meijabaum (10). Deoxyribose was determined by the quantitative Dische diphenylamine reaction as modified by Racker (11), spectrophotometric readings being made at 610 mμ. It was found that crystalline 2-D-deoxyribose, its anilide, and purified dicyclohexylammonium deoxyribose 1-phosphate (DCHDR 1-P) all gave identical results, as based on their deoxyribose content, with this procedure. Protein nitrogen was determined by the method of Kingsley (12). The method of Friedkin (13) was used for determination of P_i in the presence of the very labile ester,

DR1-P. In this procedure, aliquots of the enzyme reaction mixtures were suitably diluted and centrifuged to clarify them in order to avoid protein precipitation when determining the concentration of precipitated magnesium ammonium phosphate by the Gomori (14) method. The method of Lowry and Lopez (15) was used to determine P_i in presence of R1-P; the 0.125 *M* pH 4.0 acetate buffer employed contained 0.05 μM./ml. of $CuSO_4$ (16). In this procedure interference of protein was eliminated by chilling the samples to 0° C., adding the reagents at this temperature, and promptly centrifuging them at 8000 *g* before making the spectrophotometric determinations. R1-P phosphorus was determined as that labile in 0.5 *N* HCl in 10 minutes at 26° C. (17).

Preparation of Enzyme

Muscle of lingcod in strictly fresh condition, and usually in *rigor mortis*, was used. Satisfactory preparations were obtained from fish which had been frozen and stored for over a year at −20° C. All procedures, unless otherwise stated, were conducted at 0° to 3° C. The muscle was blended for about 1 minute with three volumes of water and sufficient 1 *N* HCl to adjust the mixture to pH 6.0. Insoluble protein was removed by centrifuging 45 minutes at 2500 *g*. The supernatant liquid was filtered through glass wool to remove floating particles, and the filtrate made 60% saturated with ammonium sulphate at pH 7.0. The precipitate, collected by centrifuging as above, was suspended in a small amount of water and dialyzed 2 to 3 hours against running water (4° to 8° C.) and finally against successive changes of demineralized water until practically free from ammonium sulphate. The resulting crude preparations contained 3 to 7.5 mg./ml. of protein N. They proved satisfactory for preparation of R1-P, DR1-P (see below), and deoxyribose 1,5-diphosphate (18).

The crude preparations were purified as follows: Amberlite XE 64 cation exchange resin was prepared and conditioned either by the method of Hirs, Moore, and Stein (19), or that of Boardman and Partridge (20). The columns used were 5.3 cm. in diameter and contained a coarse sintered glass disk covered with a layer of fine glass wool. They were filled to a height of approximately 20 cm. with resin equilibrated with 0.2 *M* phosphate buffer pH 5.5. Denatured protein was removed from crude enzyme preparations by centrifuging 10 minutes at 25,000 *g*, and from 100 to 200 ml. (500 to 1800 mg. of protein N) was placed on a resin column and eluted, usually under slight pressure (about 10 cm. of Hg) with phosphate buffer similar to that used to condition the resin. Tests showed that nucleoside phosphorylase activity, as determined by formation of R1-P from guanosine (Fig. 1) closely paralleled the protein concentration in fractions collected from the columns. The protein-containing fractions were pooled (0.11 to 0.5 mg./ml. protein N), adjusted to pH 6.8, dialyzed against 0.5 *M* NaCl until free from P_i, freeze-dried at about 10 μ pressure until they contained between 0.5 and 2.0 mg./ml. of protein N, and stored at −20° C. Table I shows that the resin purification step removed 93% to 96% of the protein from the crude preparations.

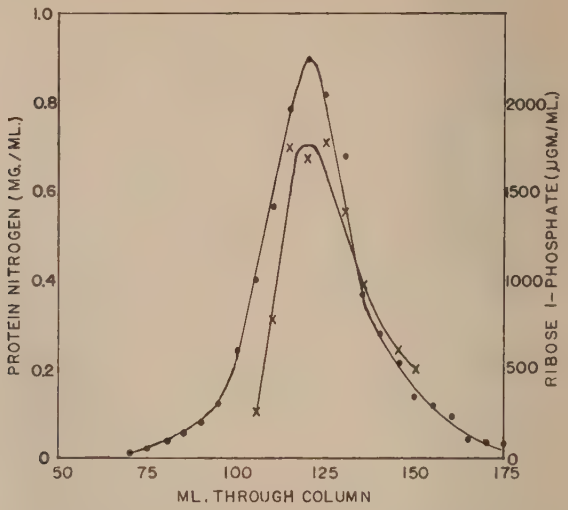

FIG. 1. Protein nitrogen content (●——●) and phosphorylase activity (×——×) of 5 ml. fractions of nucleoside phosphorylase from an XE 64 cation exchange resin column. The phosphorylase activity was determined by incubating small aliquots of the fractions at pH 7.2 and 37° C. for 2.5 hours in presence of 10 μM./ml. each of guanosine and P_i. The R1-P content was determined by quantitative paper chromatography.

TABLE I

REMOVAL OF PROTEIN FROM CRUDE NUCLEOSIDE PHOSPHORYLASE BY PASSAGE THROUGH AMBERLITE XE 64 RESIN pH 5.5

Ml. crude enzyme applied	Mg. protein applied	Ml. eluate collected	Mg. protein in eluate	% protein removed by resin
220	1620	280	104	93.5
200	1400	260	101	92.8
135	505	166	23.2	95.5
100	650	235	26.1	96.0

The comparative activity of several crude and purified preparations, certain of which had been stored for between 1 and 2 years at about −20° C., was determined. The preparations were diluted with 0.05 *M* veronal buffer pH 7.4, so that they contained very different protein concentrations. Tubes containing 0.5 ml. portions of the various enzymes and 10 μM./ml. each of R1-P and hypoxanthine were incubated at 37° C., development of P_i being followed at 30-, 60-, and 120-minute intervals using 30 μl. aliquots. The rate of P_i formation was approximately linear under these conditions. The results (Fig. 2) show that the purified preparations were about three times as active on a protein basis as the crude preparations. Since about 5% of the total protein of the crude preparations was recovered after the resin treatment, the loss of over-all activity in this purification procedure was about 75%. The data from Fig. 2 indicate that the activity of the purified preparations is 32.3

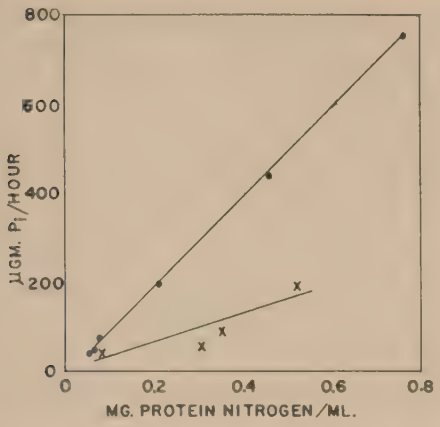

FIG. 2. Orthophosphate liberation from R1-P in presence of hypoxanthine by different concentrations of crude (×——×) and purified (●——●) nucleoside phosphorylase.

μM. P_i/mg. protein N/hour. When four purified preparations made from fresh, or from frozen fish stored over a year at −20° C., were similarly tested using lower substrate concentrations (5 μM./ml. each of R1-P and hypoxanthine), the average activity expressed as above was 20.3 μM. These results compare favorably with those obtained by Kalckar (21) for liver purine nucleoside phosphorylase (14.7 μM. hypoxanthine/mg. protein N/hour at 25° C.) and by Friedkin and Roberts (22) for thymidine phosphorylase (42.7 μM. thymidine/mg. protein N/hour at 38° C.), but are considerably lower than those obtained by Rowen and Kornberg (23) for their highly purified nicotinamide riboside phosphorylase (255 μM. nicotinamide riboside/mg. protein N/hour at 38° C.).

Several attempts were made to purify the enzyme further. Fractionation with cold acetone or ethanol (24) occasioned marked loss in activity and no appreciable concentration in any of the fractions. Attempts at fractionation at 3° C. on XE 64 resin conditioned with phosphate at pH 6.1, 6.6, and 7.05 were ineffective. The single protein fraction which emerged from an XE 64 resin column buffered to pH 5.7 with 0.2 *M* sodium citrate was totally devoid of nucleoside phosphorylase activity.

Two major protein zones were evident when small amounts of purified preparations were subjected to electrophoresis (Fig. 3). When comparatively large amounts of enzyme (e.g. 40 μg. protein N) were so treated two or three minor zones were evident. Evidence was obtained which indicated that the phosphorylase activity was associated with the fast moving protein component. However, electrophoresis on paper, or in starch blocks, yielded preparations which only possessed extremely slight activity.

Two purified preparations (containing 2.0 and 2.1 mg./ml. of protein N) were examined at 260,000 g in a model E Spinco analytical centrifuge by Dr. J. Polglase of the University of British Columbia. The results showed that they

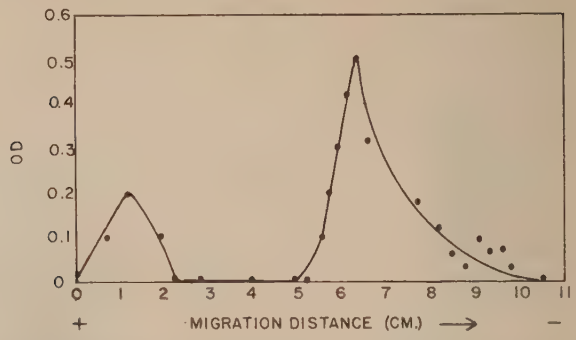

FIG. 3. Electrophoretic pattern of purified nucleoside phosphorylase. The preparation (8 μg. protein N in 50 μl.) was applied to Whatman 3 MM paper, and subjected to electrophoresis in 0.1 *M* sodium veronal buffer, pH 8.6 (18 hr.; 14 v./cm.; 3° C.).

contained two major protein components which were only partly separated. Crude and purified preparations possess phosphoribomutase activity which, unlike that in mammalian liver, cannot be removed by surface denaturation (25).

Preparation of Deoxyribose 1-Phosphate

The method of Friedkin and Roberts (22) was employed initially. In a typical experiment 0.5 mM. each of deoxyguanosine and Na_2HPO_4 were added to 5 ml. of purified nucleoside phosphorylase (2 mg./ml. protein N) and incubated 6 hours at 37° with occasional agitation. The pH, which slowly increases owing to enzymic deamination of guanine, was kept between 7.0 and 7.8 by occasional cautious addition of small portions of 1 *N* HCl. Crystalline DCH DR1-P (101 mg.) was obtained which, on recrystallization, yielded 72 mg. of a product in which the content of deoxyribose and of phosphorus labile in 100 minutes at pH 4.0 (13) was 98% of the theoretical. It was found later that the ester could be obtained in higher yield when crude nucleoside phosphorylase enzyme was used as follows.

In a typical experiment 1 mM. each of deoxyguanosine and Na_2HPO_4 were mixed with 20 ml. of crude enzyme (3.9 mg./ml. of protein N), the pH being adjusted initially to 7.4 with 1 *N* HCl. The mixture was stirred rapidly with a magnetic stirrer at 36° C., the pH being adjusted as in the above experiment. The increase in DR1-P was measured by determining the decrease in P_i at intervals, and it was found that this reached a maximum after 7 hours. The mixture was kept overnight at 0° C., centrifuged at 8000 *g*, and the residue resuspended and washed with 25 ml. of water. The combined supernatant liquids were adjusted to pH 9.5 with 1 *N* NaOH and absorbed on a 15×3 cm. (diam.) column of Dowex 1×10 resin in the formate cycle. The column was washed with 200 ml. of water and then eluted at 3 ml./minute with 0.2 *M* NaCl, 10 ml. fractions being collected. Fractions 13–21 inclusive, which gave a strongly positive cysteine – sulphuric acid test (26) for deoxyribose, were pooled and an excess (2 mM.) of crystalline barium acetate added. The

solution was evaporated to dryness *in vacuo* using a flash evaporator (bath temperature 37° C.). The residue was extracted with several successive portions of warm water, the insoluble barium salts being removed by centrifuging. To the clear supernatant liquid (7 ml.) four volumes of 99% ethanol were added slowly, and the solution chilled for several hours at 0° C. The precipitate was collected by centrifuging, washed with 99% ethanol, and dried 3 hours over P_2O_5 in high vacuum at room temperature. The yield was 360 mg. of Ba DR1-P, which was approximately 65% pure as determined by its content of deoxyribose and phosphorus labile at pH 4.0.

The crude barium salt was dissolved in 15 ml. of water and run slowly through a 10×0.65 cm. (diam.) column of Dowex 50×8 cation exchange resin in the cyclohexylamine cycle, the column being washed with water (35 ml.) until the eluate no longer gave a positive test for deoxyribose. The clear solution was evaporated to dryness as above. The white residue was dissolved in 7 ml. of water-saturated *n*-butanol, the solution filtered through a small Whatman No. 1 conical filter to remove traces of insoluble material, and the paper washed with three successive 1-ml. portions of water-saturated *n*-butanol. To the clear filtrate and washings anhydrous diethyl ether was added slowly at 0° C. until slight clouding occurred. After crystallization began more ether was added slowly to a total volume of 30 ml. The mixture was held 1 hour at 0° C. The crystalline residue was collected on a small tared medium porosity Pyrex sintered glass filter, washed with anhydrous ether, and dried 3 hours over P_2O_5 in high vacuum. The yield was 212 mg. (0.52 mM.) of DCH DR1-P, C, 48.66; H, 8.89; calc. for dicyclohexylammonium deoxyribose 1-phosphate $C_{17}H_{37}O_7N_2P$ (M.W. 412.46), C, 49.5; H, 9.04. The deoxyribose content was 103% of theoretical. Phosphorus liberated in 100 minutes at 20° C. in 0.063 *M* acetate buffer pH 4.0 was 98% of the theoretical amount.

In a second similar experiment, using another crude enzyme preparation, 2 mM. of deoxyguanosine yielded 840 mg. of Ba DR1-P of only approximately 50% purity based on its deoxyribose and labile phosphorus content. In this experiment the dicyclohexylammonium salt was crystallized from anhydrous methanol and ether, and 440 mg. (1.07 mM.) of pure DCH DR1-P were obtained. C, 49.45; H, 9.20; $[\alpha]_D^{22.5°} = +38.76°$ (*c*, 2.57 in H_2O). The optical rotation of this natural DR1-P is similar to that reported by Tener *et al.* (6) for synthetic dicyclohexylammonium α R1-P ($[\alpha]_D^{20} = 40.3°$), which is the form which is enzymically active. This, in addition to the data concerning its enzymic activity, strongly suggests that natural DR1-P possesses the α configuration. Wright and Khorana (5) have shown that β R1-P is laevorotatory ($[\alpha]_D^{23°} = -9.28°$).

Preparation of Ribose 1-Phosphate

The poorer yields of this ester in comparison with those obtained for DR1-P are explained by the fact that the phosphoribomutase activity of the crude nucleoside phosphorylase preparations is apparently considerably greater than

that of the phosphodeoxyribomutase activity. However, satisfactory preparations of R1-P were obtained by the following procedures: (*a*) Guanosine and Na_2HPO_4 (1.75 mM. of each) were mixed with 50 ml. of crude riboside phosphorylase (2.1 mg./ml. protein N) and incubated 6 hours at 37° C. with occasional agitation. Ba R1-P of 95% purity, as judged by its content of ribose and of phosphorus labile in 0.1 *N* perchloric acid at 23° (25), was obtained using the original isolation procedure described by Kalckar (21). The yield (150 mg.) was 0.39 mM. calculated as the monohydrate, M.W. 384.5 (5). (*b*) Guanosine and Na_2HPO_4 (0.88 mM. of each) were incubated 6.5 hours with 10 ml. of purified riboside phosphorylase (2.1 mg./ml. protein N). The R1-P was prepared by the low temperature chromatographic procedure of Rowen and Kornberg (23) and isolated as the barium salt. The yield was 118 mg. and the purity was 76% as assessed by its content of ribose and labile phosphorus. The recovery was therefore 0.23 mM. (calculated on the basis of the pure compound). Experience has shown that these impure barium salts rarely yield products of high purity by simple reprecipitation of their aqueous solutions by addition of ethanol. (*c*) Guanosine and Na_2HPO_4 (2 mM. of each) were stirred at 36° C. with 40 ml. of crude riboside phosphorylase (3.9 mg./ml. protein N), the pH being maintained between 7.0 and 7.8 by cautious addition of 1 *N* HCl. R1-P formation, as determined by the decrease in P_i, reached a maximum after about 4.5 hours. At this stage chromatography showed that R1-P, and some R5-P, were present. The reaction mixture was adjusted to pH 10.5 with 2.5 ml. of 2.5 *N* NaOH, the protein coagulated, and the R5-P largely removed by heating to 80°, and the clear cooled centrifuged solution treated by the same general procedure as used for DR1-P, including absorption on a Dowex 1 formate column, elution with 0.2 *M* NaCl, and pooling of the fractions which gave a strongly positive orcinol reaction. Excess barium acetate was added to the pooled eluates, followed by addition of an equal volume of 99% ethanol. The barium salt of R1-P was extracted with water and reprecipitated with ethanol. The yield was 295 mg. which was 63% pure as assessed by its content of ribose and phosphate labile in 0.5 *N* HCl in 10 minutes at 26° C. (17). DCH R1-P was prepared from the crude barium salt by the procedure used with barium DR1-P. The dried eluate obtained from the Dowex 50 cyclohexylamine resin column was dissolved in 5 ml. of water-saturated *n*-butanol, filtered, and anhydrous diethyl ether (15 ml.) was added slowly at 0° C. until crystallization occurred. After washing and drying as with DCH DR1-P, 0.34 mM. (145 mg.) of crystalline salt was obtained. C, 46.95; H, 8.64; P, 7.10. Calc. for $C_{17}H_{37}O_8N_2P$ (M.W. 428.46): C, 47.65; H, 8.71; P, 7.23. The ribose content was 98% of the theoretical. The preparation contained 0.7% P_i.

Results

Activity and pH

Solutions containing purified nucleoside phosphorylase (0.045 mg./ml. protein N), R1-P (2.5 μM./ml.), hypoxanthine (5 μM./ml.), and the desired buffer (0.04 *M*) were prepared (0.2 ml. volume) and incubated, with occasional

gentle shaking in small stoppered tubes, in a water bath at 37° C. Samples (70 μl.) were removed after 1 hour for determination of orthophosphate. The results (Fig. 4) show that the enzyme has a wide range at which it exerts about the same activity (pH 5.5 to almost 7.5). There is a very sharp decrease in activity below pH 5.5 and a somewhat slower decrease between about pH 7.2 and 9.2.

Rather similar results were obtained in an experiment in which 0.2 ml. portions of purified enzyme (0.11 mg./ml. protein N), the pH values of which were adjusted initially to the desired values with NaOH or HCl, were incubated 4 hours at 37° C. with 10 μM./ml. each of guanosine and P_i. The amounts of R1-P present in the various samples were then determined by quantitative paper chromatography. The results of this experiment indicated that the activity was most pronounced between pH 6.8 and 7.4 (Fig. 4).

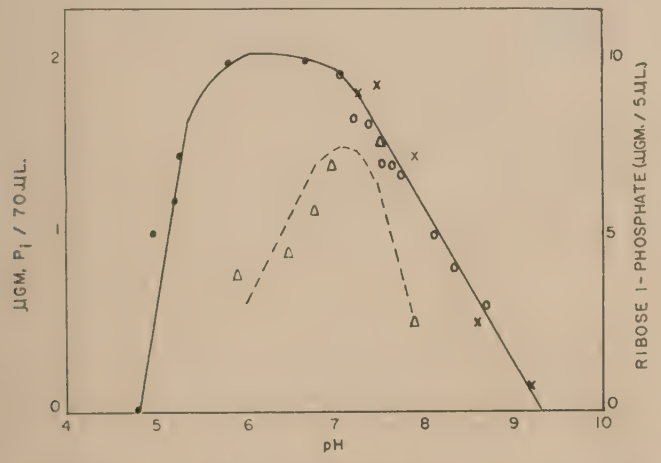

FIG. 4. pH activity curves of purified nucleoside phosphorylase. Sodium succinate (● ——●); tris(hydroxymethyl)aminomethane-HCl (○ — ○); and sodium veronal (× ——×) buffers with hypoxanthine and R1-P: P_i increase determined. Quantitative paper chromatographic determination of R1-P formed from guanosine and P_i (△——△).

Pentose Specificity

Small stoppered tubes containing 0.4 ml. of purified enzyme (0.37 mg./ml. protein N), 5 μM./ml. each of the pentose phosphates listed in Table II, 0.1 ml. of 0.1 *M* veronal buffer pH 7.4, and an excess (10 μM./ml.) of hypoxanthine were incubated at 37° with occasional agitation. The increase in orthophosphate in 50-μl. aliquots was determined at intervals as an indication of inosine synthesis. The results (Table II) show clearly that only natural R1-P and synthetic α R1-P acted as phosphate donors in this system, and that β R1-P and arabinose 1-phosphate were not utilized. These findings show, as has already been pointed out (3, 5, 6), that phosphorolytic cleavage of β-nucleosides is accompanied by an inversion of C-1 of the ribofuranose molecule to yield α R1-P.

TABLE II

FORMATION OF INOSINE FROM HYPOXANTHINE BY PURIFIED NUCLEOSIDE PHOSPHORYLASE IN PRESENCE OF PENTOSE 1-PHOSPHATE ESTERS

	Per cent inosine formed as calculated from orthophosphate liberated after:			
	3 min.	30 min.	60 min.	120 min.
Natural R1-P	12.8	83.5	89	90
Synthetic α R1-P	11.6	75.5	86	87
Synthetic β R1-P	0	0	0	0
Synthetic arabinose 1-P	0	0	0	0

TABLE III

PURINE SPECIFICITY OF PURIFIED NUCLEOSIDE PHOSPHORYLASE PREPARATIONS

		P_i from R1-P after:		P_i from DR1-P after:	
Substrate*	Enzyme preparation†	3 hr.	6 hr.	3 hr.	6 hr.
Controls		0	0	0	0
Hypoxanthine	*a*	78	81	67	63
	b	85		62	
	c	80			
Xanthine	*a*	29	36	43	45
	b	43	54	39	
	c	19			
6-Mercaptopurine	*a*	3.9	6.5	42	46
		5.2	6.1		
	b	28	33	38	
6-Methylpurine	*a*	0	5.2	0	3.2
	b	0	7.8	2.5	
8-Azaguanine	*a*	37	67.70	28	41
	b	37	45	41	
	c	24			
Guanine	*a*	7.8	11	8.7	14
	b	16	19	5.1	
	c	19			
Adenine	*a*	3.6	7.7	9.7	10
	b	19	31	14	
	c	13			
2,6-Diaminopurine	*a*	<1	2.3, 2.6	0	0
	b	12	20	4.5	
	c	9			

*Purine, isoguanine, 2-aminopurine, 2-oxypurine, 2-mercaptopurine, 2-thioadenine, 2-thioguanine, 8-azaxanthine, 8-chloroxanthine, 8-aza-6-oxy-2-thiopurine, 4-amino-5-imidazolecarboxamide, uric acid, uracil, cytosine, and thymine were all inactive.

†The final concentration of enzyme present in the different experiments was (mg./ml. protein N): (*a*) 0.44; (*b*) 0.42; (*c*) 0.46.

Purine Specificity

The various purine or pyrimidine compounds were dissolved, or suspended, in 0.05 *M* veronal buffer, pH 7.4, to make solutions containing 25 μM./ml. Where necessary the pH was adjusted with 1 *N* NaOH. Solution of the more insoluble compounds could be facilitated in some instances by heating. Small stoppered tubes received 0.1 ml. of purine or pyrimidine solution and 0.4 ml. of purified nucleoside phosphorylase in which the R1-P or DR1-P had been dissolved in 6.25 μM./ml. concentration. The samples were incubated at 37° C. with occasional gentle agitation, 100-μl. aliquots being withdrawn at intervals for determination of P_i by the appropriate method.

The results obtained in several experiments using different enzyme preparations are given in Table III. They show that the enzyme had the same general specificity toward purines when either DR1-P or R1-P was used as substrate. In general, hypoxanthine, xanthine, 8-azaguanine, 6-mercaptopurine, and guanine were strongly activated. Adenine and 2,6-diaminopurine were also activated to a lesser degree. Only compounds with a substitution in the C-6 position, and none of those substituted in the C-2 position, were utilized. None of the pyrimidines was activated. The reason why 8-azaguanine was utilized, while 8-azaxanthine was not, has not been explained. No hypoxanthine was detected by paper chromatography in reaction mixtures which contained adenine and, since adenosine was detected in such chromatograms, it may be assumed that the adenine is activated without previous deamination to hypoxanthine.

Equilibrium

The first studies concerning a nucleoside phosphorylase enzyme showed that the equilibrium indicated in the following system was about 90% in favor of nucleoside formation: hypoxanthine+R1-P$\rightleftharpoons$inosine+P_i (21). Investigations concerning equilibrium in enzyme systems similar to the above are often complicated by the slight solubility of many nucleosides and their corresponding bases in water. With the hypoxanthine-inosine system the solubility of the components is sufficient to ensure quite rapid equilibrium when the experiment is initiated with either hypoxanthine plus R1-P (DR1-P) or with inosine (deoxyinosine) plus P_i. Thus when these reactants in 10 μM./ml. concentrations were incubated with nucleoside phosphorylase (0.75 mg. ml. protein N) under conditions similar to those used in previous experiments, the equilibrium was about 85% in favor of nucleoside formation, as is shown for R1-P in Fig. 5. Almost identical results were obtained with the DR1-P in the deoxyinosine system.

A similar experiment was carried out in which 10 μM. each of guanosine and Na_2HPO_4 were stirred at 36° C. with 2 ml. of purified enzyme containing 0.83 mg. ml. of protein N, the pH being maintained between 7.2 and 7.8. With these concentrations of reactants there was no apparent undissolved purine or nucleoside at equilibrium. P_i, phosphorus labile in 10 minutes in 0.5 *N* HCl at 20° (R1-P phosphorus), and phosphorus labile in 10 minutes in 1 *N* NaOH at 100° C., were measured at intervals in 50-μl. aliquots. Since

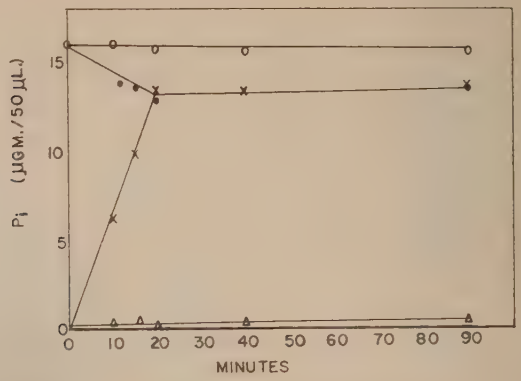

FIG. 5. Equilibrium with purified nucleoside phosphorylase in the hypoxanthine–inosine system. P_i+enzyme (○——○); P_i+enzyme+inosine (●——●); R1-P+enzyme (△——△); R1-P+enzyme+hypoxanthine (×——×).

it has been found that 58% of the phosphorus of R5-P is liberated by heating in 1 *N* NaOH as above, while R1-P is stable under these conditions (5), this factor was used to calculate P_i arising from R5-P. The results (Table IV) show that after 3 hours 75% of the phosphorus was in the form of P_i. Thus the equilibrium was in favor of nucleoside formation. This experiment was somewhat complicated since the purified enzyme possessed phosphoribomutase activity, though this only became marked toward the end of the experiment. Both R1-P and R5-P were demonstrated chromatographically (solvent *b*) in samples taken after 7 hours' incubation.

In another experiment, using the same purified enzyme preparation, guanosine and orthophosphate were added in 50 µM./ml. concentration. In this case equilibrium was attained after 12 hours with 50% each of P_i and labile phosphorus (R1-P), and this remained unchanged even after 18 hours incubation. It is interesting to note that, with these comparatively high concentrations of reactants, no R5-P could be detected by paper chromatography, a result which is in accord with the above phosphate determinations. These

TABLE IV

EQUILIBRIUM BETWEEN GUANOSINE+P_i AND GUANINE+R1-P USING PURIFIED NUCLEOSIDE PHOSPHORYLASE

Time at 36°, hours	µg. P recovered in 50-µl. aliquots as:*			
	P_i	R1-P	R-5P	Total P recovered
0	15.5	0	0	15.5
2	13.0	—	—	—
3	11.5	—	—	—
5	11.1	2.4	1.7	15.20
7	11.7	2.25	2.0	15.55

*Total P present = 15.5 µg./50 µl. aliquot.

results, which show that R1-P synthesis is favored by high concentrations of guanosine, serve to explain why it is possible to obtain quite good yields of the phosphate ester without removing the end product (guanine), as is essential when inosine is used (21).

Proof of Unity of the Enzyme

Tubes containing an excess (50 μM./ml.) of hypoxanthine, purified enzyme (diluted with 0.05 *M* veronal buffer pH 7.4 so that the final concentration in the reaction mixtures was 0.022 mg./ml. of protein N), and 20 or 40 μM./ml. of R1-P or DR1-P (alone or combined) were incubated with frequent agitation. The concentration of orthophosphate in 50-μl. aliquots was determined at intervals (15). The results (Table V) show that, under conditions where the enzyme was apparently saturated with respect to substrates, a mixture of R1-P and DR1-P occasioned about the same rate of nucleoside synthesis as did either ester alone in the same molar concentration. This indicates that R1-P and DR1-P are both activated by the same enzyme.

TABLE V

PHOSPHORYLATION OF HYPOXANTHINE (50 μM./ml.) BY PURIFIED NUCLEOSIDE PHOSPHORYLASE SATURATED WITH RESPECT TO R1-P, DR1-P, AND A MIXTURE OF THESE ESTERS

	μg. orthophosphate in 50 μl. after:		
	0.5 hr.	1.0 hr.	2.0 hr.
DR1-P, 20 μM./ml.	5.7	8.4	10.0
R1-P, 20 μM./ml.	5.2	7.2	9.8
DR1-P+R1-P, 20 μM./ml.	6.5	8.8	9.5
DR1-P, 40 μM./ml.	6.3	9.0	10.1
R1-P, 40 μM./ml.	5.7	7.4	10.5
DR1-P+R1-P, 40 μM./ml.	6.5	9.0	11.4

Heat Destruction

The purified enzyme preparation used had been prepared from muscle frozen 3 months at −20° C., and had itself been stored 1 month at this temperature before use. It was diluted with an equal volume of 0.05 *M* veronal buffer pH 7.4 and portions heated at 50° C. for different lengths of time before its

TABLE VI

PER CENT P LIBERATED AS P_i FROM PENTOSE PHOSPHATES BY PHOSPHORYLASE PREHEATED AT 50° FOR INTERVALS NOTED

	Minutes preheated					
	0	30	90	180	300	420
Substrates	P_i liberated					
R1-P + hypoxanthine	87	85	87	87	47	16
DR1-P + hypoxanthine	83	81	83	81	61	18

activity was determined. The conditions employed were similar to those described in the foregoing experiment, except that the final concentration of enzyme was 0.125 mg./ml. of protein N. P_i was determined after 2 hours' incubation using 100-μl. aliquots. The results (Table VI) show that slow inactivation occurs at 50° C.

Effect of Calcium, Magnesium, and Cysteine

No significant acceleration or depression of the activity of the purified enzyme in hypoxanthine-inosine system was apparent following the addition of 0.01 or 0.001 *M* Mg^{++}, Ca^{++}, or cysteine, or mixtures of these.

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Some Sociological Effects of Quota Control of Fisheries

by

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Recently striking changes have occurred in many of our fisheries. Some of them seem to centre around the management of the fisheries by quota. Most of these changes, when not forced upon us by circumstances, have been economically advantageous. They have, however, strongly affected in one way or another the lives of the people engaged in the fishery. Superficially some of the results do not appear to be improvements. If the well-being of the people is in fact impaired by quota control, it may be appropriate to examine the sociological implications of this tool of management. The following comments point out some of the problems. Some of these may seem to justify investigation by sociologists.

Quota management of fisheries is resorted to in cases where we presumably know how much fish can be removed from a stock without diminishing the contribution of the stock to our economy. It is applied by allowing fishing from an opening date until the amount of the pre-determined quota is caught up. Apparently where we have the required biological information, quotas provide a seemingly logical method of management for maintaining fish stocks at levels where their yield is maximum or otherwise optimum.

Any type of management will have economic and sociological ramifications. Catch quotas are no exceptions. And as in the cases of other means of management economic and sociological effects are inextricably mixed.

My interest in the sociological results of quota regulation was first roused at a public hearing on halibut regulations for the International Fisheries Commission. As background, I might mention that quota regulation of halibut had been followed by a great increase in availability of fish which led to very fast fishing. The good fishing attracted additional fishing efforts. As a result of the high reward for fishing effort, and of the increased effort, the quotas were (and are still) caught up very quickly. The incident involved the protest of a veteran Norwegian skipper. I wish I could accurately reproduce in full his dignified but perturbed statement. Instead I shall have to provide a poor paraphrase —something like this: "They say that the halibut is being conserved and perhaps it is. I don't know. I do know that before the halibut was conserved and I worked on the schooners I used to fish halibut nine months of the year and make a decent living. Now the season is so short a boat can't catch enough fish to provide a year's income. So I have to turn to working on salmon packers and other things that don't suit me."

So much for our Norwegian friend. He said enough to make it clear that he had suffered severe vocational dislocation as a result of management policies imposed to increase the growth of the halibut biomass. For him it was an important matter.

It seems that sociological stresses result from quota regulation because the regulation accentuates competition among fishermen on the grounds. In some cases there

FRB No. 527

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are co-operative aspects to operations within fishing fleets so that for any situation of fish density and distribution there may be an optimum amount of fishing effort. However, fishermen are always in competition with each other. The fish caught and boated by one fisherman are no longer available to his fellows. However, under quota regulation competitive effects are reinforced. The fish landed by one fisherman reduce the chances of his competitors (and of himself, too, for that matter) of catching the fish which remain in the water. The situation could be approximated under some circumstances where fishermen were in competition for markets, but such occurrences are not common in stabilized fisheries.

The competition leads to an hectic scramble for fish, inordinately long hard working days, perhaps taking unwise chances, near financial disaster to the individual from breakdowns in gear and equipment or from temporary illness. To me these things do not seem good from the point of view of the fishermen involved.

It is more difficult to assess the effects of concentrated production on the economics of fishing. While it seems likely that much uneconomic expense is incurred for competition, there must be real advantage in the mechanization that is encouraged.

The situation for packers and secondary handlers of fish and their shore crews is also difficult to assess. To do so one must weigh the advantages of concentrated predictable operating seasons and their evident economies and rewards against the disadvantages of general rush, crowded facilities, and overworked crews. The effects of congestion and delay on the quality of the fish placed before the consumer might also be considered as a sociological result.

The main sociological effect of quotas would appear to be the discouragement of specialized full time fishermen or of fishermen who specialize in one particular fishery. The fisherman is forced into other fisheries or other occupations in order to maintain his position in society. To some very worthy people this is a most unwelcome development. Whether it is actually bad or not would appear to be a problem for the sociologists. If bad, it must be decided whether to endure the shortcomings so as to enjoy the advantages of a tidy method of management, or to modify the management method to adapt it to human needs.

Another and related effect arises from the very productive fishing each year at the beginning of the quota period. This encourages many fishermen to take part in harvesting the resource. Some of them are part time fishermen or men who are only moderately well qualified for the work. Their participation adds money and variety to their own lives. The fish they catch, however, are taken away from the potential landings of professional fishermen.

Some of the foregoing remarks may suggest basic antagonism to quotas as a method of management. There is no such antagonism. Management of a fishery is complicated and none of the applicable tools is perfect. I recognize that quotas also have shortcomings.

We must realize also that quota control shows strongly features which are present in some other devices of management. For example, seasons, whether imposed by regulation or by the availability of the fish, when the size of the exploitable stock is limited, may have similar effects.

In closing I might add that trial has shown that the headlong exploitation stimulated by quotas even has some disadvantages from the point of view of maintaining the fish stock. The halibut fishery—opening intensively at the beginning of the growing season for the fish—derives little advantage from the growth of the current year. In addition intensive fishing for halibut takes the quota before all parts of the stock are fully available and thus failed to make complete use of the resource until special adjustments were invoked. To me it seems that these biological defects in the quota system resemble what I regard as the sociological shortcomings. Each defect arises from ignoring the complexities of its subject matter.

Edmond Cloutier, C.M.G., O.A., D.S.P., Queen's Printer and Controller of Stationery, Ottawa, 1958

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Regulation of the Lobster Fishery

by

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Introduction

Canada's lobster fishery is our most valuable inshore fishery on the Atlantic Coast. In recent years the catch has approximated 50 million pounds with a marketed value of 20 million dollars. Almost 20 thousand fishermen fish about two million traps valued at over five million dollars. In the major producing areas inshore fishermen derive a high proportion of their fisheries income from this one species.

Everyone is agreed that conservation of this valuable resource is of primary importance but just what is conservation and what are its objectives? Conservation has been defined as "Wise Use" and a commonly stated objective is to obtain the maximum sustained yield. In marine fisheries the yield is usually thought of in pounds. With staple foods, sufficient production to maintain national nutritional standards is, of course, of primary importance. The lobster, however, is a luxury item that contributes relatively little to our nutritional welfare. Even if our total production were consumed in Canada the per capita consumption of edible meat would be less than one pound per year. The per capita consumption in North America is less than two ounces a year. With such a product it can be questioned whether the maximum sustained yield is as important an objective as the welfare of the 20,000 primary producers.

Regulations

Over the 100 year history of the lobster fishery numerous regulations have been adopted. These have become so complex in detail as to require a special office consolidation for ready reference. Of these regulations the three most important appear to be those that (a) prohibit the sale of egg-bearing lobsters, (b) establish closed fishing seasons and (c) provide for a minimum legal size. To what extent have these regulations affected the yield from the fishery or the welfare of the fishermen?

Protection of egg-bearing lobsters

Lobsters mature when 7 to 12 inches long and the females spawn every other year. The eggs remain fastened to the abdominal swimmerets until they hatch a year later. Regulations prohibiting the sale of egg-bearing lobsters are in general supported by fishermen, particularly in areas where such lobsters are scarce.

Observations in the southern Gulf of St. Lawrence over the past nine years on the newly-hatched, free-swimming larvae show that the hatch varies as much as 2:1. Survival during the one to two month free-swimming period is, however, much more variable – the numbers surviving to the last free-swimming stage varying as much as

FRB No. 528

58514-1—1

40:1. To date it has not been possible in this area to demonstrate any clear relationship between the number hatched and the number that survive the free-swimming period. In this area lobsters mature early with the result that egg-bearing females and larvae are abundant—apparently many times as abundant as in southern Nova Scotia, an area of late maturity where very few lobsters escape the fishery to become mature. In spite of this great disparity in the abundance of mature lobsters and larvae, the commercial production in the two regions is very similar—about 10,000 pounds per square nautical mile. These observations have failed to provide any factual basis for protecting egg-bearing females. Although the regulation may be of little value in improving the yield or the welfare of the fishermen it seems unlikely that it is actually harmful. The regulation is popular with most fishermen, it affects a relatively small proportion of the catch, and the lobsters released become legal when the eggs hatch.

Closed seasons

Closed seasons were originally introduced in an effort to reduce the fishing intensity and so arrest a steady decline in landings during the early 1900's. Later, adjustments in the seasons were made to aid marketing and to permit fishermen to engage in other fisheries or other seasonal occupations. At present in the Bay of Fundy and southern Nova Scotia the season is open for six to $7\frac{1}{2}$ months but along the remainder of the coast for only two to three months. Studies of tag returns, size compositions, seasonal declines in catch per unit effort, days fished, gear set and hauled show that the rate of exploitation is often higher in the short seasons. The fishery has simply adjusted to the shorter season by employing more men, boats and gear. It seems obvious that closed seasons have failed in their primary objective of improving the sustained yield. They have, however, had numerous secondary effects—some good, some bad.

With short seasons fishermen can turn to other fisheries, farming, lumbering etc., and in some areas it is only by so doing that they are able to subsist. Closed seasons have improved the quality of the lobsters in that less than 20 per cent are caught from July to September when they are soft-shelled and slack meated, difficult to hold and ship and give a poor meat yield. Closed seasons are difficult and costly to enforce and supplementary regulations restricting fishermen, boats and gear to one fishing season a year have been adopted. These supplementary regulations have been seriously questioned on sociological and economic grounds. With short seasons persons who have been gainfully employed elsewhere find it advantageous to engage in the fishery when lobsters are unusually abundant. This effectively reduces the catch of the steady fishermen who find it difficult to see the value of restrictive conservation measures when the accruing benefits are divided among individuals who have not contributed.

With year round fishing over half our lobsters would be landed from July to September and would depress an already low summer market resulting from heavy United States production. One possible solution is a universal closed season from July to September to avoid the period of heavy United States production and to

limit the sale of newly-moulted lobsters. With such a season weather and ice conditions would permit from four to nine months' fishing in the various districts. Landings would be concentrated in the early fall.

Size limits

Minimum legal sizes are established with the objective of increasing the sustained yield, either by allowing more animals to mature and so increasing reproduction or by taking advantage of the period where growth is rapid enough to more than offset losses through natural mortality. There is so little evidence of a relationship between the abundance of mature lobsters and commercial production that the prospects of size limits improving the yield through their effect on reproduction do not appear good. There is, however, considerable evidence that sub-legal lobsters grow enough to more than compensate for natural losses. Extensive marking experiments have shown that lobsters near present size limits survive well and as a general rule moult once a year and grow about 50 per cent in weight. In certain warm water areas growth of the smaller catchable lobsters exceeds 100 per cent a year. Size distribution studies show exceptionally high total mortality rates. Extremely high tag returns (general average of 60 per cent) and the rapid seasonal drop in catch per unit effort show that most of the mortality can be ascribed to the very intensive fishery. These observations provide strong support for minimum size limits. There is also the fact that wherever size limits have been observed the commercial catch has improved. On the basis of the available evidence it is concluded that size limits are the most effective means of increasing the yield from the fishery. If this conclusion is substantiated by controlled experiments, work should be extended to determine the best minimum size limits for the major stocks of lobsters.

Economic and sociological effects

There are two market categories for lobsters, the smaller "canners" which are hermetically sealed or processed as chilled meat and the larger "markets" which are sold alive. There is usually a marked price differential with the markets worth considerably more per pound to the fishermen. From the fishermen's point of view the maximum sustained yield should be measured in value rather than weight. There is, of course, the contribution that the actual processing of canner lobsters makes to the communities. Is this contribution great enough to compensate for the lower price to the primary producer?

In the final analysis conservation is action by individuals. It is the individual fisherman who releases undersized or berried lobsters. To be effective, of course, conservation must be practised by all or a large majority of those using the resource. The individual or group of individuals is led to believe and perhaps has a right to believe that he will benefit. In the lobster fishery this is not always the case. In 1947 a group of fishermen at Fourchu, Nova Scotia, agreed to a regulation which required them to release their smaller lobsters to the extent of approximately half their catch. In 1948 they further agreed to limit their traps in the firm belief that they were wasting money in excess effort. The value of the catch increased rapidly to the point where new fishermen were attracted. By 1956 the fleet had increased

80 per cent and the number of traps doubled. The catch per boat has started to decline. At Tignish, Prince Edward Island, fishermen were forced in 1954 to observe an unpopular size limit. Catches have improved and in 1956 the catch per boat was the highest in recent history. Judging from 13 years' records at this port it seems certain that the fleet will increase to the point where the average catch per boat is about the same as previously.

The rather unique features of the lobster fishery—a readily available but limited supply, a heavy demand and high price, and a relatively low capital investment lead to a degree of exploitation and competition that is perhaps unequalled in marine fisheries. Long-term, enthusiastic co-operation in restrictive conservation measures seems unlikely unless fishermen receive more assurance that as individuals they will benefit. Limited licensing is a possible but unpopular solution. Less drastic measures such as increased licence fees, licence cancellation for infraction of regulations, residence requirements and restriction of government loans for lobster boats and gear might ease the situation considerably. Any reduction in boats or gear should increase the net value of the catch.

Possibly our primary objectives in the management of the lobster fishery are (1) discover and apply the minimum size limits that will yield the greatest gross value and (2) reduce the fishing intensity to increase the net value of the catch and give the fishermen who practise conservation greater assurance that they will benefit. The problem involves a complicated interplay of biological, economic and sociological factors and is of sufficient importance to warrant detailed study.

Edmond Cloutier, C.M.G., O.A., D.S.P., Queen's Printer and Controller of Stationery, Ottawa, 1958

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Regulation of the Atlantic Salmon Fisheries

by

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Since 1949 the research and management programme for Atlantic salmon in eastern Canada has been under review by a Federal-Provincial Co-ordinating Committee. The main objective is to make more salmon available for use by both the commercial and sport fishermen. The various projects and recent results of the joint programme have been described in articles published in the April, 1955, and April, 1956, issues of the Department of Fisheries' monthly publication "Trade News".

Economic Value of the Salmon Fisheries

Wide fluctuations in total annual commercial landings of Atlantic salmon in eastern Canada have occurred since 1870 when the first statistics were published. Peak levels of production usually followed periods of below-average yields at intervals of about ten years. The most recent peak in commercial production occurred in 1930, however, when over 13 million pounds were landed, with a value of over $4 million. This was followed by a steady decline to a level of 4 million pounds in 1945, no indication of significant improvement by 1949, and a still lower level of 3 million pounds with a landed value of about $1 million by 1955. From one-half to two-thirds of these total landings have come from Newfoundland, including Labrador. The landings in all parts of the Canadian Atlantic coast have shown a similar trend, and it is interesting that there is a close correspondence between fluctuations in commercial landings here and in the eastern part of the North Atlantic around the British Isles.

There has been widespread concern over this last prolonged depression in commercial landings with its widespread effect on the netsmen, and anxiety about the future of the valuable sport fishery in the rivers. Actually there is no evidence of a general decline in angling catches since 1930, and even more salmon have likely been caught through an increased total rod effort in recent years. Since 1949 improved statistics have been obtained on angling catches, and it is known that at least 75,000 salmon are now taken annually in all the rivers combined. Of these about 45,000 are angled in the rivers of the Gulf of St. Lawrence, 5,000 in rivers around the outer coast of Nova Scotia, 5,000 in Bay of Fundy rivers, 5,000 in Quebec rivers on the north shore of the St. Lawrence, and 15,000 in Newfoundland rivers. Unfortunately it is very difficult to assign a reliable economic value to the sport fishery, where the value of recreation alone accounts for such a large proportion of the total. Judging by the value of comparable sport fisheries in the United States recently estimated by a careful census, our salmon angling is probably worth several million dollars. There is no doubt that the availability of Atlantic salmon for angling in Canadian rivers is a very valuable national asset. This, combined with the commercial fishery, which can be

FRB No. 529

58513-3—1

expected to be worth about $3 million in normal years, justifies considerable effort to see that the best possible management techniques are employed for this species.

Salmon Life History in Relation to the Fisheries in a Typical River Area

The accompanying figure shows the life history of Atlantic salmon and its relationship to the commercial and sport fisheries in a typical large New Brunswick river system. Over the whole Atlantic coast of Canada, the duration of the fresh water stage varies more than is indicated for this one area.

Spawning

A convenient starting point is the spawning of adults in October and November. Experiments on the Pollett River, N.B., are now indicating that a rather modest number of parents is needed for optimum smolt production If predatory birds, chiefly mergansers, are controlled it is estimated that about 45 pounds of adult females are needed per average mile of stream 10 yards wide, to give the optimum number of about 200 eggs per 100 square yards of bottom. This applies only to streams which commonly have two-year smolts. For rivers like the Miramichi with typically three-year smolts, about 250 eggs per 100 square yards seem to be needed. This allows for normal fishing effort after the adults have entered fresh water, so the numbers of eggs are higher than are actually required to be deposited in the spawning redds. Studies over the past five years indicate that the Miramichi River has been receiving about this level of spawners.

An important point is that a much lower egg deposition can be allowed if mergansers are not controlled, because these predatory birds will drastically reduce the number of parr to give much lower smolt production than would otherwise occur. To allow for the above levels of spawning without predatory bird control would be expected to lead to a waste of adult salmon.

From eggs to smolts

In the Pollett River experiments now in progress, the survival rate from eggs to fry with three "light" spawnings has averaged 6 per cent, while the survival rate from fry to large, pre-smolt, parr has been 80 per cent. Comparable data for "medium" and "heavy" spawnings are not yet available. It is hoped that the results of these studies combined with similar observations on other rivers will shortly give a relationship between smolt production, egg deposition, and numbers of spawning adults.

From smolts to adults

There are inadequate data yet on the rate of survival from descending smolts to the adult stage when the salmon are taken by various fisheries or contribute to the spawning escapement. Some evidence that the survival rate lies between five and ten per cent has come from our smolt marking experiments and from other investigations. For the Miramichi River the estimated total annual smolt production in recent years has averaged about $1\frac{1}{2}$ million. With such a high mortality in the sea, only 75,000 to 100,000 of this output would survive for use by the fisheries or for spawning.

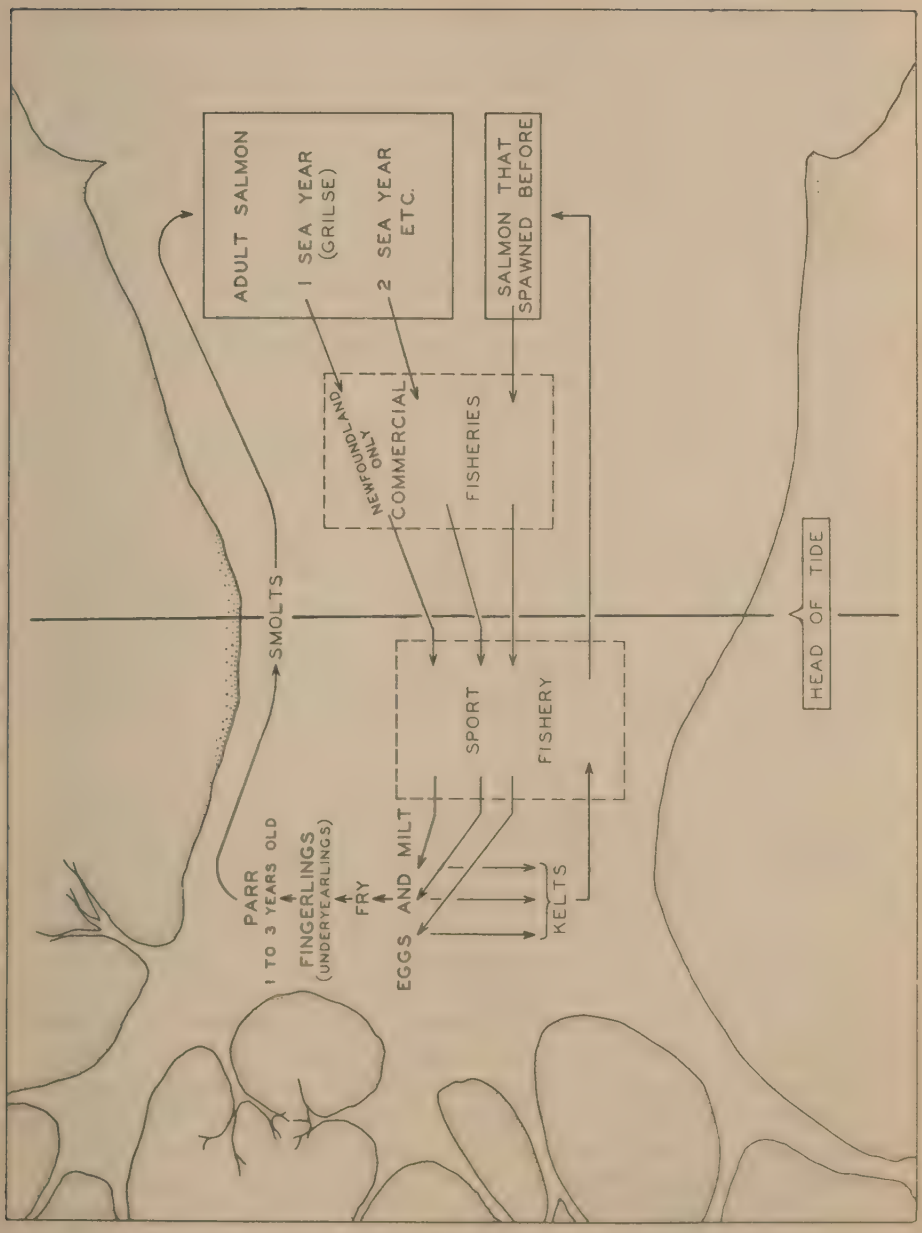

Figure 1.—Life cycle and utilization of Atlantic salmon in New Brunswick, Canada.

Capture by commercial gear

For the whole Canadian coast the gear comprises surface drift-nets operated offshore, inshore fixed trap nets and a few floating gill-nets, while some salmon are taken incidentally in gear set for other species. Traditionally the nets have been set to take adult fish as they apparently move from the feeding areas which are often far away in the sea, towards the native streams to spawn. Reliable information on the capture of adult salmon of known river origin by such gear over a wide area of the coast is now coming from a marking programme involving smolts produced in the Miramichi and Pollett Rivers, N.B., the Port Daniel River, Que., and the Little Codroy River, Nfld. It has been found, for example, that many salmon produced in Maritime streams are taken far away in commercial nets, particularly around the east coast of Newfoundland. Also they may wander into the estuaries of other maritime rivers and be caught there by commercial gear. Of course, many are taken also by the gear set in the estuary of their native river.

There are fishery regulations covering the opening and closing dates of the seasons which are different for different areas of the coast, mesh regulations aimed at protecting small salmon (grilse), weekly close periods to allow fish to escape the nets completely for a couple of days each week, but no quota systems. Most of the catch consists of two-sea-year fish, except around Newfoundland where grilse can still be caught for market in commercial nets. Each year the run of fish into many estuaries is marked by two peaks, one early in the summer and one in the autumn. In some rivers like the Miramichi the late run has by far the most fish, which come in after the commercial season is over. Only early run fish are desired by most commercial fishermen, and at present there is no demand for a fall fishery. As the spawning season approaches, salmon are not considered to be in good condition for the market.

The sport fishery

Upon reaching fresh water areas of the rivers the salmon become available to anglers, who can legally keep both grilse and large salmon. In some rivers, like the Miramichi, grilse comprise about one-half the total angling catch. Other rivers, like those of the east coast of Newfoundland, may provide only grilse angling, while in others large salmon provide the bulk of the angling catch as in some Quebec streams. In New Brunswick alone there is a legal fishery for kelts in the spring. There seems to be no objection to this on biological grounds because very few kelts (5 to 10 per cent) ever turn up again after the first spawning. About 5,000 kelts are taken each spring in the Miramichi River which is now the most popular place for such fishing.

The fishery regulations for angling include opening and closing dates for the season which differ from one area to another depending on the usual time of arrival of the fish, daily and weekly bag limits, and restriction of method to the use of artificial flies when angling for salmon.

Discussion

The primary aim of fishery regulations for Atlantic salmon is to assure an adequate spawning escapement. Obviously most of the existing regulations were put into effect many years ago when requirements for spawning were unknown. Reliable

information on this point is now being obtained for the experimental rivers where concentrated work is in progress. It is hoped that the requirements for many streams can be developed from data obtained on a few typical ones. A useful supplement to these studies is the counting of ascending fish through traps installed in many fishways, and annual surveys of spawning activities on a number of rivers now being carried out by the Conservation and Development Service of the Department of Fisheries.

It seems reasonable to aim at providing, through regulation, for the amount of spawn that will give the optimum number of young under average conditions for hatching and survival. An idea of the amount of variation to be expected from year to year in the smolt production of the same river area is provided by data of recent experiments on the Pollett River. On a ten-mile experimental stretch, ample stocking was provided by planting 250,000 fingerlings in five successive years, with predatory birds controlled. The average smolt production from these five plantings was 20,000; the minimum was 14,000, the maximum was 25,000. This variation amounts to about plus or minus 25 per cent of the basic "capacity" of the stretch to produce smolts.

A very important point concerning regulations to insure adequate spawning escapement, is the possibility of early-run or late-run adults tending to produce young salmon that will inherit the tendency to return to fresh water early or late, as adults. If this were the case and if attention were given only to the numbers of young salmon being produced per unit area of stream, the future supply of early-run adult salmon could be inadequate. There is some evidence that such a tendency to return early or late is not generally inherited in Atlantic salmon. Nevertheless, in 1954 the fishery regulations for both commercial and sport fishing were amended to allow more early-run salmon to reach the spawning grounds. It was reasoned that this could do no harm and that it was desirable to be on the safe side until more information could be obtained. This information will start to accumulate in 1957 as the first returns of adult salmon come from an experiment started in 1953. The experiment involves the planting of marked fingerlings of known parentage, in streams which have runs predominantly of the other type as regards time of entry of adults. The time of return of the marked salmon will be compared with the time of return of the native stock.

Most of the fishery regulations for Atlantic salmon have likely been too restrictive in the past, owing to inadequate knowledge. An example of a situation where liberalization of regulations might be desirable is the closing date of the commercial fishing season in the Miramichi River area. Closing the trap-net season on August 31 prevents utilization of most of the total run of salmon here each year, because at least 85 per cent of all the large salmon enter the estuary after September 1. Since 1954, however, abnormal conditions affecting smolt production have been created in the whole Miramichi watershed through extensive spraying of the woodland with DDT against the spruce budworm. The spraying has had such serious effects on parr survival that it is not safe to reduce spawning stocks at this time.

Several years ago when the present research and management programme was being planned it was necessary to assign priorities to a list of projects considered to be worthy of attention in the near future. Almost all the projects have been undertaken

except physiological and behaviour studies which would require special facilities and staff. Quite recently it has become apparent that particular attention should be given to the effects of hydro-electric developments, pollution as from mining operations or DDT spraying, deforestation, and other factors associated with industrialization, on the general behaviour and survival of various stages of salmon. New studies along these lines are now being planned. It is quite possible that the information so obtained will be of great importance in any attempts to manage the Atlantic salmon fisheries by regulation or other means a few years hence.

Edmond Cloutier, C.M.G., O.A., D.S.P., Queen's Printer and Controller of Stationery, Ottawa, 1958

SOME NOTES ON A COLLECTION OF POLYCHAETA FROM THE NORTHEAST PACIFIC SOUTH OF LATITUDE 32° N.[1]

E. Berkeley and C. Berkeley

Abstract

Twenty-nine species of Polychaeta collected on, or near, the west coast of Lower California and of Mexico are recorded. Seventeen of them are pelagic. Of these, 10 are heteronereid phases of various Nereidae, in two of which cases (*Perinereis monterea* and *Nereis oligohalina*) neither of the heteronereid phases has been described previously. One species recorded (*Maupasia caeca*) is new to the northeast Pacific.

Introduction

The polychaeta with which these notes deal were collected by Mr. W. L. Klawe of the Inter-American Tuna Commission in 1955, 1956, and 1957 and submitted to us for identification. Many of them come from the west coast of Lower California and, of these, several have already been reported from the region (18), but a large number of the earlier records are from within the Gulf of California. Some special interest attaches to this collection because of the large proportion of heteronereids and other pelagic forms comprised in it. These resulted largely from Mr. Klawe's practice of collecting at night under a light whenever opportunity offered. In the notes which follow, references are given to descriptions of each species from which the synonymy and further references may be obtained.

Amphinomidae

Chloeia viridis Schmarda. Monro (23)

Two specimens taken under a light at Los Frailes, Mexico. Both are about 50 mm. long and 12 mm. wide as preserved. They agree with Monro's description except that the dorsal markings are indistinct. Differentiation from *C. pinnata* Moore and *C. entypa* Chamberlin, the other two species recorded from the northeastern Pacific, is based on the absence of any trace of a purplish or dusky spot associated with the lateral tentacles (23). No spurred setae could be found. Serrations on the notosetae in the median region of the body are, in most cases, quite heavy.

We have previously recorded the present species, and *C. pinata* Moore, from Lower California (1), differentiating them as above, but we still entertain the doubt as to the validity of the separation which we then expressed. It has been extensively recorded from the region by Hartman (18). In our 1939 paper we recorded its capture at a night light off Galapagos Island, and Hartman (18) mentions a similar instance at Agua Verde Bay, Gulf of California. It is interesting to note again this response to a light stimulus, particularly as there was no evidence of its association with sexual maturity.

[1]Manuscript received February 19, 1958.
Contribution from the Fisheries Research Board of Canada, Biological Station, Nanaimo, B.C.

Glyceridae

Hemipodus californiensis Hartman. Hartman (17)

A single specimen, collected on a sandy beach at Ensenada, Lower California, agrees completely with the original description. This was based on material collected on the coast of California. The only subsequent record until the present one seems to be that of Rioja (31) from Mazatalan, Mexico.

Alciopidae

Callizona angelini (Kinberg). Fauvel (12)

Four pelagic specimens, taken, respectively, at the following stations: (1) 10° 03′ N. lat., 88° 52′ W. long., (2) 6° 55′ N. lat., 83° 38′ W. long., (3) 4° 16′ N. lat., 83° 56′ W. long., (4) 3° 41′ N. lat., 83° 00′ W. long., are typical of the species.

The species does not appear to have been recorded previously, as such, from localities so near to the equator. *Rhynchonerella pycnocera* Chamberlin was, however, taken at several stations in the vicinity by the Carnegie Expedition of 1928–1929 (39) and the characters of that species (6) are close enough to those of *Callizona angelini* to suggest their identity, as Monro (22) has already done.

Chamberlin (6) gives grounds for considering that the generic name *Rhynchonerella* should be substituted for *Callizona*, but we are inclined to agree with Monro (22) in thinking that the adoption of this synonymy "would only lead to confusion".

Greefia oahuensis McIntosh. Wesenberg-Lund (41)

A specimen taken in surface plankton at 9° 03′ N. lat., 104 °34′ W. long., is incomplete posteriorly. It measures 35 mm. for 55 segments and is 7 mm. wide. The ventral pedal glands are colorless, as described by Monro (22, 25). The species was originally recorded from Honolulu. This is the first subsequent Pacific record.

Corynocephalus paumotanus Chamberlin. Chamberlin (6)

Four specimens in plankton at 10° 52′ N. lat., 88° 02′ W. long., average about 10 mm. in length and 2 mm. in width. The species was taken by the Carnegie Expedition of 1928–29 in the same neighborhood (39).

Phyllodocidae

Lopadorhynchus (subgen. *Lopadorhynchus* s. str.) *uncinatus* Fauvel. Fauvel (12)

A single specimen taken in a plankton tow from 450 m. at 20° 00′ N. lat., 110° 35′ W. long. The specimen is 16 mm. long, about 3.5 mm. wide, and consists of 30 segments. It agrees with Fauvel's description in all respects except that the termination of the ventral cirri in parapodia posterior to the third is much more extended than described and illustrated (12, Fig. 67*e*) resembling in this respect the condition found in *Lopadorhynchus* (subgen. *Prolopadorhynchus*) *appendiculatus* (35). No pigment remains in the eyes.

Dales' record of the species from Monterey Bay, California (8), was the first from the Pacific. The present one extends the distribution considerably further south; Treadwell (39) records *L. nans* Chamberlin and *L. varius* Treadwell from approximately the same latitude as the present specimen. These are both distinguishable from *L. uncinatus* by the presence of simple setae in some parapodia other than the first three. Moreover, *L. nans* has three anterior segments with exclusively simple setae, whilst *L. uncinatus* has only two. The former species is regarded by Wesenberg-Lund (41) as synonymous with *L. brevis* Grube.

Lopadorhynchus (subgen. *Lopadorhynchus*) *brevis* Grube. Wesenberg-Lund (41, p. 12)

Two specimens taken in surface plankton at 3° 03′ N. lat., 101° 35′ W. long., and at 10° 52′ N. lat., 88° 02′ W. long., respectively, agree with Wesenberg-Lund's description. One is 12 mm. long and 4 mm. wide and consists of 25 segments, the other somewhat smaller. The species is regarded by Wesenberg-Lund and by Monro (22, p. 78) as synonymous with *L. nans* Chamberlin which has been recorded by Treadwell (39, p. 33, and Map 1, p. 56) from approximately the same latitude and both east and west of the present record.

Maupasia caeca Viguier. Fauvel (12, p. 190)

In surface plankton at 1° 58′ N. lat., 83° 49′ W. long. A single specimen 6 mm. long, about 1.5 mm. wide (without setae), and consisting of 17 setigers. The only previous record of the species in the Pacific is that of Ushakov (40, p. 268) from 43° 08′ N. lat., 156° 08′ E. long.

Nereidae

All the Nereidae in the collection except *Nereis* (*Neanthes*) *succinea*, were taken in the heteronereid phase (see Introduction). In many cases the identification of species in this phase is difficult owing to the modification of the posterior parapodia for swimming and has to be based largely on the paragnaths and the number of anterior setigers without swimming setae. In the key to the species occurring in the collection these characters are used as far as possible. Most of the species recorded were males. Figures given in boldface at the end of each entry in the key indicate the number of setigers anterior to the first epitokous foot in the male.

Key to Species

1. Paragnaths absent; three-regional body; 16–34 ..... *Nicon mexicana*
   Paragnaths present 2
2. All paragnaths conical 3
   Some paragnaths bars 8
3. All paragnaths present 4
   Group V of paragnaths absent 5
4. Anterior notopodia with three lobes; 15–16 ..... *Nereis* (*Neanthes*) *succinea*
   Anterior notopodia with two lobes. 16–17 ..... *Nereis* (*Neanthes*) *oligohalina*
5. Paragnaths of Group VI, four or five large cones 6
   Paragnaths of Group VI, small points 7

6. Paragnaths of Groups VII and VIII, several irregular rows with the largest ones in the anterior row; 14–20 ..... *Nereis* (*Nereis*) *pelagica*
   Paragnaths of Groups VII and VIII, several irregular rows, all of nearly equal size excepting some smaller ones on the anterior part of VII; 14 ..... *Nereis* (*Nereis*) *grubei*
7. Paragnaths of Group VI, five to nine in two rows; 14 ..... *Nereis* (*Nereis*) *veleronis*
   Paragnaths of Group VI, six to ten in oval groups; 15–30 ..... *Nereis* (*Nereis*) *zonata*
8. Paragnaths of VI, smooth bars; 22 ..... *Perinereis monterea*
   Paragnaths of VI, pectinated **9**
9. Paragnaths of Groups I, II, and V absent **10**
   Paragnaths of Groups I, II, and V present; 14 ..... *Pseudonereis gallapagensis*
10. Simple heavy falcigers in some anterior parapodia; 16–25 ..... *Platynereis dumerilii* var. *agassizi*
    No simple heavy falcigers; 14 ..... *Platynereis polyscalma*

Nicon mexicana (Treadwell). Treadwell (38, p. 1 as *Leptonereis*), Hartman (20)

Two specimens taken at a night light off shore at Marina Madre Island, Mexico. Both are three-regional male heteronereids and appear to be identical; one is, however, so small that examination is difficult. The other is 25 mm. long and agrees well with the descriptions of the species except that there are fewer anterior setigers without swimming setae than is indicated (40 instead of "at least 55"). Hartman (20) altered Treadwell's generic designation from *Leptonereis* to *Nicon* on the ground that the unchanged posterior parapodia have no foliaceous dorsal lobes. Claparède (7) and Fauvel (12) include *Nicon* in *Leptonereis*.

The original, and only, previous records of this species are from stations in the Gulf of California, in the general neighborhood, but considerably farther north, than the present one.

Nereis (*Neanthes*) *succinea* (Leuckhart). Fauvel (12)

Two specimens, both in atokous phase, found on driftwood. The first, taken off Colombia at 6° 35′ N. lat., 78° 03′ W. long., is 22 mm. long, the second taken off Rio Anton, Gulf of Panama, is about 10 mm. long. Both agree with descriptions except that no paragnath can be detected for Group V on the smaller one. The species was recorded from the coast of Guatemala and that of Costa Rica by Ehlers (10, p. 118, as *N. acutifolia*), from the Panama region by Monro (23), and from the Gulf of California by Rioja (33).

Nereis (*Neanthes*) *oligohalina* Rioja. Rioja (32)

Four male heteronereids taken at a light at Hipolito Bay, Lower California, varying in length from 12 mm. to 15 mm., correspond closely with Rioja's description, so far as agreement can be determined in the heteronereid phase. The characters of the prostomium and those of the anterior parapodia posterior to the seventh agree with his figures (32, Figs. 13 and 15). The arrangement of the paragnaths is in accordance with his description as amended (34). There are 17 anterior setigers without swimming setae. The anterior 12 to 14 setigers have irregular dark spots which, jointly, form three or more longitudinal lines. Rioja's records are from brackish waters in the Gulf of Mexico.

Nereis (*Nereis*) *pelagica* Linné. Fauvel (12)

Four male heteronereids taken at a light in Hippolito Bay, Lower California, two about 20 mm. long, the others about 14 mm., agree with this species

except that there are only 14 anterior setigers without swimming setae instead of the more usual 16. A cosmopolitan species.

Nereis (*Nereis*) *grubei* Kinberg. Reish (30)

A single male heteronereid from Ensenada, Lower California, is typical. The species has been recorded under a variety of synonyms from California (30). It has also been recorded from the Gulf of California and from the west coast of Mexico (as *N. pseudoneanthes* Hartman) by Rioja (34).

Nereis (*Nereis*) *veleronis* Hartman. Hartman (18)

Three male heteronereids, taken at lights. Two specimens, from Abreojo Point, Lower California, are about 20 mm. long, the third, from Guayquil Bank, Ecuador, is a little larger. All agree with Hartman's description. The homogomph notopodial falcigers which are present in the atokous phase of the species (18, Fig. 73) do not appear until about the 30th setiger and are excluded from the epitoke. The species is recorded previously from Peru.

? *Nereis* (*Nereis*) *zonata* Malmgren var. *persica* Fauvel. Fauvel (11)

Six male heteronereids ranging in length from 3.5 mm. to 1.5 mm. taken at Hipolito Bay, Lower California, at a light, agree in respect of the paragnaths, the shape of the anterior setigers without swimming setae, and the form of the specialized dorsal homogomph falciger with the atokous phase of *N. zonata*. This falciger occurs in the present specimens as far forward as the 30th setiger, where the change to the heteronereid foot has already begun. The only character which disagrees with descriptions of the epitoke of *N. zonata* is the position in the body at which the change to the epitokous foot occurs. Instead of only 15 setigers without swimming setae in the anterior region, the specimens have 30 or more.

N. zonata is characteristically a northern species, but Fauvel (14) describes the variety *persica* from the Persian Gulf, which he says (11) scarcely differs from the type. We refer the present specimens as above with some hesitation.

Perinereis monterea (Chamberlin). Chamberlin (5)

Five specimens of the female heteronereid from Los Frailes, Mexico, ranging in length from 25 to 50 mm., and one of the male from Cedros Island, South Bay, Lower California, 40 mm. long; all taken at night lights. Neither of the heteronereid phases of the species has been recorded previously. There are 23 anterior setigers without swimming setae in the female, 22 in the male. The atoke was originally reported from Monterey Bay, California, and since from several Californian stations. It is recorded from Mexico by Rioja (31).

Pseudonereis gallapagensis Kinberg. Gravier (16), Hartman (18)

A single specimen of the male heteronereid, 10 mm. long, taken at a light at Sihuatanejo, Mexico. The species is widely known from the region covered in this paper.

Platynereis dumerilii Audouin and Milne-Edwards var. *agassizi*. Berkeley and Berkeley (3)

Six male heteronereids taken at lights. One at Hipolito Bay, Lower California, the remainder at Cedros Island, Lower California. This variety occurs widely in the northeast Pacific north of the region dealt with in this paper. Most of the previous records are of atokes, but the epitokes have been described. Rioja (34) records the atokous form from the south coast of Lower California and from stations in the Gulf of California.

Platynereis polyscalma Chamberlin. Chamberlin (6), Hartman (18)

Three male heteronereids about 25 mm. long taken at a light at Los Frailes, Mexico. Chamberlin described only the epitokes and these have been taken more recently in widely separated regions. The present specimens agree with Chamberlin's description of the male except that we find 14, instead of 15, anterior setigers without swimming setae. Hartman (18) finds that the change occurs in the male "after the 13th segment". She describes the atokous phase for the first time from the Gulf of California and the south coast of Lower California, and epitokes from Ecuador.

Eunicidae

Eunice enteles Chamberlin. Chamberlin (5)

A single example dredged in 15 fathoms at Los Frailes, Mexico, is 40 mm. long and about 3 mm. wide at the widest region of the body. Branchiae begin on the 4th setiger with one filament, increase to a maximum of five filaments at the 9th, and soon thereafter decrease gradually until there are only two at the 25th setiger. There is only one filament on the 30th setiger and this condition persists for only a few segments. Thereafter only the dorsal cirrus remains to the end of the body. These branchial characters lead us to attribute the specimen to *E. enteles*, rather than to *E. antennata* Savigny, which it resembles in other respects. Hartman (19) believes these two species to be synonymous and it is possible that the differences noted may be a matter of age. *E. enteles* is recorded previously from the west coast of Mexico by Rioja (31).

Arabella semimaculata (Moore). Moore (27, as *Aracoda*), Berkeley and Berkeley (2)

A single specimen from Turtle Bay, Lower California, collected on a rocky shore, is 160 mm. long and about 4 mm. wide. The species is recorded widely from California. Hartman (19) says it extends south to Peru.

Lumbrinereis latreilli Audouin and Milne-Edwards. Fauvel (12), Hartman (19)

An example of this species dredged in 15 fathoms at Los Frailes, Mexico, is in three pieces. A cosmopolitan form.

Cirratulidae

Cirriformia (*Audouinia*) *luxuriosa* Moore. Moore (26, as *Cirratulus*); E. and C. Berkeley (2)

Two specimens from Turtle Bay, Lower California, both collected intertidally under rocks. One is 60 mm. long and about 6 mm. wide, the other slightly smaller. The species is widely recorded from California. Rioja lists it from Lower California (31, 33).

Caulleriella alata (Southern). Southern (36, as *Chaetozone*), E. and C. Berkeley (4)

Two small specimens, dredged in 15 fathoms at Los Frailes, Mexico. Both are small, poorly preserved, and each is in two parts. The incidence of bidentate crotchets with wings in the most anterior neuropodia suggests the species *alata* which we have identified in material from Pacific Grove, California (unpublished).

Sabellariidae

Idanthyrsus pennatus (Peters). Johansson (21), Okuda (28)

A specimen from coral at Santiago Bay, Mexico, 40 mm. long, excluding the caudal region. The species is recorded widely from coral reefs.

Sabellidae

Branchiomma mushaensis Gravier. Monro (24)

A specimen collected at Ceralbo Island, Gulf of California, labelled as having been taken "from the shell of a living pearl oyster" is typical of this species. It has been recorded previously from California (2) and from the Gulf of California (15, 33, as *Megalomma*).

Dasychone cingulata Grube. Johansson (21, as *Branchiomma*), Okuda (29)

Several specimens taken from the bait-well of the M. V. May-Queen, the vessel from which the material dealt with in this paper was collected. The species is widely distributed in warmer seas.

Serpulidae

Hydroides uncinata (Phillipi). Fauvel (13), Ehlers (9, as *Eupomatus*)

Specimens varying from 10 to 15 mm. in length, collected at Turtle Bay, Lower California, under rocks. The species is recorded by Treadwell (37, as *Eupomatus*) and by ourselves (2) from California, and from the Panama region by Monro (24).

Pomatoceros minutus Rioja. Rioja (31, 33)

Several specimens from floating seaweed at Abreojo Point, Lower California. The species has been recorded from a number of Mexican stations by Rioja.

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AMINO ACID ANALYSIS BY HORIZONTAL PAPER CHROMATOGRAPHY

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INTRODUCTION

e advantages of circular paper chromatography have been discussed by several thors[1-5] and could be confirmed by our initial trials, notably with respect to well fined sharp bands obtained with this method. They contrast favourably with the fuse spots frequently observed with the two-dimensional technique[6]. Besides, the cular technique permits the comparison of different proteins or different hydrolytic ctions on one and the same paper[7,8]. GIRI and co-workers[8] had achieved this on linary chromatographic paper but the K.C.T. technique[9], in which a paper with e machine made slits is used, constitutes an additional improvement.

In confirmation of GIRI and co-workers[5] it was found that the solvent system butanol acetic acid water (4:1:5) causes a number of amino acids to run in pairs that nineteen amino acids are separated into nine groups. These authors proposed o methods for separating the groups into their individual constituents. The first e[5] consists of rechromatographing the sample up to 6 times, the second one[10] of ting the group bands by capillary action onto another paper and developing same th another solvent, *e.g.* pyridine water (80:20), pyridine isoamyl alcohol water :10:7) or pyridine amyl acetate water (10:5:5). KRISHNAMURTHY AND SWAMI-THAN[11] had proposed a third method, in which they used five different solvent stems, determining from each chromatogram only those amino acids which are parated into a single and homogeneous band. RAO AND WADHWANI[12,13] also use eral solvent systems but could reduce the number of these to three by adopting RI's method of rechromatography.

The conventional circular method, in which the spot is applied near the centre d in which the solvent system *n*-butanol acetic acid water (4:1:5) is employed did our experience not separate the lysine histidine group adequately from the arginine baragine group and even the next band, aspartic acid glycine serine frequently s too near the preceding one. This point is of particular importance, because the ation of the groups which are to be eluted and chromatographed with another vent system can only be determined from their ninhydrin-developed counterparts another segment of the circle, but the calendering of the paper often produces a vent front of oval shape instead of an ideal circle, so that instead of the desired nd only, parts of the neighbouring bands also are easily cut out. Probably for similar

p. 326.

reasons GIRI[14] emphasized the need for increasing the size of filter paper from 15 35 cm in one of his later publications.

Neither modifications in the size of the paper tongue (0.3 to 1.0 cm) nor replaci same by a capillary[9], alleviated the difficulty mentioned above. Thus the proble was formulated as follows: (a) to find a solvent system and other conditions, whi would separate 18 amino acids into a small number of well defined and well separat groups, (b) in which the amino acids are distributed in such a pattern that they c be easily separated by using not more than two other solvent systems, so that maximum number of groups can be eluted and chromatographed on one and t same K. C. T. paper.

PRELIMINARY EXPERIMENTS

Initial trials with butanol–acetic acid–water (4:1:5), *tert.*-butanol–formic acid–wat (75:0.8:24.2), collidine–water, lutidine–water, phenol–water and phenol–isoprop alcohol–water (70:5:25) failed to meet all requirements postulated above. The failu with phenolic systems, in spite of vacuum distillation, NaCN-addition to the chambe purification with petroleum ether–water[15], and aluminium treatment[16] was especial disappointing, since these solvents produce by the ascending or descending techniq R_F values which differ widely from those obtained with other solvent systems. Initi trials with butanone–water appeared promising, but the highest R_F values were low that even after three rechromatograms the groups were too crowded. Pyridi additions to this system gave better results but the solvent system *n*-butano butanone–water–ammonia 17 *N* (5:3:1:1), proposed by WOLFE[17] for two-dimension procedures appeared most suitable and was used for a more detailed investigatio

Chemicals

n-Butyl alcohol, AnalaR, B.D.H.
2-Butanone, Eastman Kodak.
Ammonia, concentrated, analytical quality.
tert.-Butanol, Eastman Kodak.
2,4-Lutidine, refined, Koopers Co., Ltd., Pittsburgh, Pa.
Formic acid, 85–90%, Fisher Certified Reagent.

All organic solvents, except formic acid, were redistilled, those of boiling poin over 100° *in vacuo*.

Solvent systems

n-Butanol–butanone–water–ammonia conc. (5:3:1:1, v/v), *tert.*-butanol formic aci water (75:0.8:24.2, v/v) and lutidine–water (2:1, v/v) give one phase only when th constituents are mixed.

Paper

Sheets of Whatman chromatographic paper No. 1 were used for the separations in 6 groups. They were washed according the procedure of KAY and co-workers[18] a

References p. 326.

into circles of 34 cm diameter. With a double razor three slits are made, producing
centre segments, each with an angle of 30° (Fig. 1).

The centre slit should run parallel to the direction in which the paper is calendered

Fig. 1

Fig. 2a

Fig. 2b

Fig. 2c

s. 1, 2a, 2b and 2c. Separation of a protein hydrolysate into six groups and of these into their ividual amino acids. 2a: S1, glutamic and aspartic acid; S3, histidine, alanine, serine. 2b: S2, ne, arginine, glycine; S4, valine, methionine, tyrosine. 2c: S5, threonine; S6, leucine, isoleucine, nylalanine. The figures 1 to 6 indicate the groups. S1–S6 the corresponding pure standard ples. K.C.T.-paper 2a and 2c have been developed with BuOH:HCOOH:H_2O, 2b with dine:H_2O. Experiments 2a and 2c can be carried out on one paper, the addition of standard samples on the same paper not being necessary, once the technique has been established.

order to avoid complications from the oval shape of the chromatogram.

Subseparation of the groups into individual amino acids was carried out on atman No. 1, K.C.T. paper of 26.5 cm diameter which contains 5 equally spaced lial slits (Figs. 2, a, b and c).

estigations

order to find such conditions as to produce the widest possible space between the

ences p. 326

TABLE I

VARIOUS MODIFICATIONS OF THE CIRCULAR AND TRIANGULAR METHOD

Expt.	*Method*	*Amount of solvent in ml**	*Number of runs*	*Length of runs = R_F 100 in mm*	*Total time required at 18–20°*	*Results*
A	Circular	35	1	140	12	Groups too crowded
B	Circular	35	2	130	24	Better than A
C	Circular	35	4	145	48	Better than A and B
D	Circular, but paper divided by 4 slits	35	1	135	13	Worse than A
E	*ditto*	35	2	150	26	Worse than B
F	*ditto*	35–12	1	160	13	Inadequate for fast moving bands
G	*ditto*	35–12	2	150	26	Inadequate for fast moving bands
H	Triangular, angle 37°	35	1	200	12	Crowded, but spacing more regular than above
J	Triangular, angle 45°	35	2	220	39	Quite good
K	Triangular, angle 37°	35–1	2	215	37	Inadequate for group of medium R_F-values
L	Triangular, angle 45°	35–5	2	180	30	Inadequate for group of medium R_F-values
M	Triangular, angle 42°	35–15	2	180	26	Widening of bands reduces spaces between them
N	Triangular, angle 30°	35	2	238	50–60	Satisfactory, 5 mm or more between the 6 groups

* The first figure indicates the amount of developing solvent. The second one additional amounts, put in separate dishes

groups obtained with the solvent system *n*-BuOH 2-butanone-NH_4OH-H_2O, ious modifications of the horizontal technique were tried (Fig. 4 and Table I).

These tests permit the following conclusions: With the circular method posed by GIRI two rechromatograms are required as a minimum (A, B). Four hromatograms (C) give the best results as regards separation but the arcs the fast moving groups extend to more than 15 cm, which is a detrimental factor the capillary elution. This difficulty may be overcome by cutting the group bands half and eluting each of them separately, which however constitutes a loss of ie.

When the circular method was modified by dividing the paper into four equal ments, similar to the K.C.T.-technique, lower R_F values were obtained than with GIRI-technique. Saturation of the atmosphere by putting dishes with additional vent in the chamber increased the R_F values, but some bands widened and the ervals were reduced. GANGULI[21] had proposed to modify the circular technique by tting the starting point near the periphery, which increases the absolute length of run. In order to prevent the widening of the bands into dimensions which render illary elution impractical, two triangular segments were made in the paper (Fig. 1). turating the atmosphere by placing additional solvent in the cabinet produced also h this modification wide bands and small spaces in between (K, L, M). The best arations were obtained with a segment angle of 30° resulting in an R_F 100 of mm (C, N). This confirms experiment J in which already quite satisfactory spacing s observed and in which R_F 100 was equal to 220 mm.

thod

e separation into 6 groups is effected in a wooden box, 37.5 · 37.5 · 5 cm, which overed by a glass plate. The inside and the upper rim are impregnated with molten raffin. The glass plate is affixed with adhesive tape. The paper is supported by 30 ss needles, vertically inserted in small pieces of cork and regularly spaced.

The solvent, 35 ml, is placed in a flat-bottomed dish, 2.5 cm high and 5 cm wide. glass tube, provided with a groove at the lower end, serves as an additional support the paper and as a leader for the paper tongue, which has a width of 1 cm. The ter is inserted into a razor slit at a distance of 5 cm from the periphery of the paper. e amino acid mixture is applied on both segments at a distance of 1 cm from the per tongue. After the spots have been allowed to dry, the chromatogram is run till solvent front reaches the end of the slits at the right and left side. At a temperature 18–20° this usually takes 22–30 h. The paper is allowed to dry, one segment is veloped with ninhydrin, the corresponding zones on the other segment are marked th a compass and cut out for capillary elution.

pillary elution and subseparation of groups

is is nearly identical to the method of GIRI AND RAO[10], except that the leaderstrip d the cut out group band are placed between two microscope slides, which are held gether with paper clips (Fig. 5). This modification reduces the time required for the

ferences p. 326.

elution process and can be accomplished in 30 instead of 90 min. Leaderstrip and cu
out group band should be of equal width.

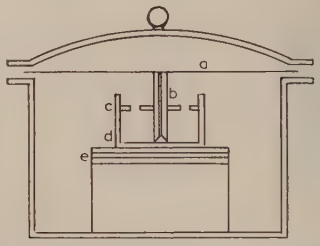


Fig. 3. Chromatography with K.C.T.-paper. a, K.C.T.-paper; b, capillary; c, perforated glass plat
resting on pieces of glass tube not shown in sketch; d, solvent vessel; e, glass plates for heig
adjustment.

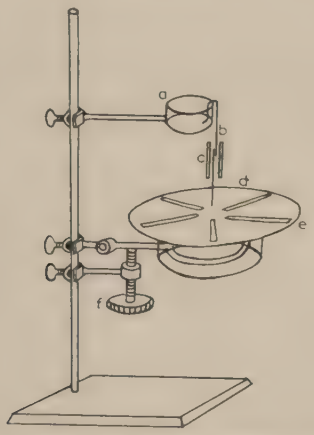
The eluting technique is critical, an excess of water may flood the paper. Two n
in the upper vessel of Fig. 5 usually gave a suitable speed which, if necessary, can b
increased by dropwise addition of water from a pipette. The K.C.T.-paper should b
in contact with the lower tip of the cut out zone. When the eluted spot has extende

Fig. 4. Spacing, width and R_F values of six groups of amino acids, obtained under various co
ditions with the solvent *n*-butanol–butanone–ammonia–water. See also Table I.

References p. 326.

4 mm diameter, the K.C.T.-paper is detached by a turn of the screw (Fig. 5), dried
3 cm distance from a hot plate for 30 sec and replaced. Fifteen to twenty repetitions
usually adequate, as proved by ninhydrin tests with the eluted paper, twenty-five
be made for good measure. Zone 1, 3 and 6 are transferred to different segments of
K.C.T. paper, which is chromatographed with *tert.*-butanol–formic acid–water
l zone 2 and 4 are eluted on different segments of another paper which is chromato-
phed with lutidine–water.

The separation of the groups into individual amino acids is carried out in desicca-
s of 28 cm width (Fig. 3). The solvent reaches the paper through a capillary, 3 by 0.6



5. Chromatography with K.C.T.-paper and capillary elution. a, solvent vessel; b, leaderstrip; c, microscope slides; d, cut out band from first chromatogram; e, K.C.T.-paper.

with a 0.36 mm bore. The capillary has a groove at its lower end, permitting access
he solvent and is held in vertical position by a perforated glass plate, which rests
3 small pieces of glass tube or small weighing glasses. The glass vessels for the
eloping solvents are identical to those used in the wooden cabinet and contain
ml of liquid. In the case of *tert.*-butanol formic acid water, 130 ml of solvent are
ed in a large petri dish at the bottom of the desiccator. This increases the speed
this slow-moving solvent system. For lutidine water the addition of 20 ml was
quate to saturate the atmosphere.

The R_F values obtained by this method differ sufficiently for cutting out and
ting the ninhydrin compounds of the various amino acids, especially as the bands
only 3 to 5 mm wide when the two solvents mentioned above are used with 133
hydrolysate (Table II). As observed by other authors, the R_F values vary to a
tain extent, but the intervals between them are practically constant. Groups 2 and
how each a tendency to split up into two bands. As regards group 2 no useful
pose would be served by cutting its bands out and eluting them separately, because
idine–water separates all four amino acids of this group adequately (Table II).
group 6 isoleucine may be separated from the last group, containing leucine and

erences p. 326.

TABLE II

SEPARATION OF A PROTEIN HYDROLYSATE INTO SIX GROUPS AND SUBSEPARATION OF THESE IN INDIVIDUAL AMINO ACIDS ($R_F \times 100$)

Group	*Amino acids*	*n-BuOH–methyl ethyl ketone–water–ammonia, 17 N (5:3:1:1), after 2 runs*	*tert.-BuOH–HCOOH–H_2O (75:0.8:24.2)*	*Lutidine–water (2:1)*
1	Cysteine		26*	
	Glutamic acid	7	39	
	Aspartic acid		48	
2	Lysine	12		17
	Arginine			25
	Glycine	15		39
	Hydroxyproline			—
3	Histidine		76	
	Alanine	20	50	
	Serine		38	
4	Tyrosine			65
	Valine	28		44
	Methionine			51
	Proline			—
5	Threonine	33	37	
6	Isoleucine–leucine	41	63	
	Phenylalanine	46	53	

* The eluted spot was treated with a spot of H_2O_2. Non observance of this will produce fro reagent cystine one more band at R_F 16. Proline[22], hydroxyproline[23] and trytophan[24] were dete mined by specific tests.

phenylalanine, but since the two leucine isomers are usually reported together, th subseparation is not absolutely necessary. If the removal of acid from the hydrolysa is inadequate, as may happen in case only one vacuum destillation has been applie some threonine may be found in the 6th group.

After the completion of each run, the chromatograms are put in a ventilate fume hood till the translucency of the paper disappears, which usually takes fi minutes. They are then washed twice with chloroform, dried at 60° and develope with ninhydrin according to the technique of KAY and co-workers[18]. Omitting th chloroform treatment of chromatograms run with lutidine may give low value especially for glycine and methionine.

The effect of temperature variations

The temperature of our laboratory usually fluctuates between 19 and 22°, which d not affect the results described here. Higher fluctuations (15–22°), which occurre occasionally on account of breakdowns of the electric supply, interfered strongl As a precaution the chromatographic chambers and desiccators may be placed inside cabinet.

DISCUSSION

The fact that difficulties have been experienced in duplicating the results of oth workers in the field does not in our opinion justify the statement of PROOM AN WOIWOD[19] who regard circular paper chromatography as "admirable for the separa tion of two or three substances, (but) not a satisfactory substitute for large sca

References p. 326

per chromatography in the separation of----protein hydrolysates". Although we lt it necessary to modify some details of the technique proposed by GIRI, we fully ree with this author's conclusion[20]: "The method is well suited to the separation, entification and even quantitative estimation of----amino acids----".

Reduced laboratory space, controlled solvent flow, small amounts of solvents, arp bands, the facts that the centre of gravity of each zone is clearly defined[3] and at reference samples can be applied to the same sheet of paper as the test sample are lvantages, which outweigh the additional step of capillary elution. It is true that certain amount of crowding occurs, since no solvent system has as yet been found lich spaces 18 amino acids evenly. Therefore it is in the opinion of the authors more portant to have a small number of widely spaced groups in the first chromatogram an to have a greater number of crowded ones, provided the eluted groups can be sily separated into their individual amino acids. For the latter purpose the pyridine-ntaining solvent systems of GIRI did not always give ideal results on K.C.T. paper, obably because its radius is smaller than that used by GIRI. The combination of lvent systems described in this paper permits adequate separations. The fact that ROOM AND WOLWOD could not confirm GIRI's results may have also been caused by fferent amounts of solvents used. The latter author mentions only a "provision, here necessary for irrigation after the atmosphere was completely saturated----by taching a glass tube with stopcock at the side of the basin"[5]. The amounts of solvents relation to the volume of the cabinets used in both laboratories would be of interest, cause our experiments G, K, L and M show that increased amounts of solvents iden the bands.

Occasionally the egg shape formation, resulting in distorted bands, made itself lversely felt with the K.C.T. paper. A typical case is shown in Fig. 2b with respect group 4. One can of course draw conclusions as regards identity from the R_F values, t if a similar paper could be manufactured with an even number of slits, for instance ur, arranged in positions vertical and parallel to the calendering of the paper, this terference would be less disturbing, because test sample and standard sample could spotted opposite each other, so that the location of each spot of a band would rrespond to that of its antipode.

Since the length of the run appears to be one of the most important factors, the iangular technique has been tried also with the solvent system *n*-butanol acetic id water (4:1:5). Ten groups are obtained after two runs, but the intervals between e of them are only 3 mm wide. This solvent system may nevertheless be suitable r proteinaceous substances, containing a limited number of amino acids only. The stribution of amino acids is under investigation and will be reported separately.

SUMMARY

Various modifications of the circular paper chromatographic method for the quantitative determination of amino acids in protein hydrolysates have been investigated. With the system *n*-butanol–2 butanone–water–ammonia (7:3:1:1) six groups are obtained. Two groups are separated by their individual components with the system butanone–water, three others with *tert*-butanol–propionic acid–water (7:1:2). Threonine appears as a single band. Length of the run is increased and separation is improved by dividing the paper into triangular segments and by putting the starting point near the periphery.

(continued on p. 326)

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SHOULD WE PRE-WRAP FRESH FILLETS IN CONSUMER PACKAGES?

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THERE HAVE BEEN many discussions and often heated arguments among fish dealers over the most efficient way to wrap and hold fresh, unfrozen fillets. Should the fillets come in direct contact with the ice used to chill them? Some experienced retail merchants answer this question: "Decidedly no! Melting ice water is one good way of leaching the flavour and many of the valuable nutrients out of the fish. It is better practice to keep the fish in a cold room away from ice altogether." Other retail merchants have expressed the direct opposite opinion: "Our secret of having good quality fish is the abundant use of good clean ice. Ice cold water is one of nature's best cooling agents, and by slowly running over the fish, the melting ice water also washes away many of the spoilage bacteria and their offensive by-products."

Arguments and mere expressions of opinion, however, are never satisfactory substitutes for facts. Both these men based their arguments on well known facts: 1) that running water will leach out both the flavour and many of the valuable nutrients from a fillet; 2) ice water is an efficient cooling agent. Furthermore many of the soluble and gaseous by-products of bacterial activity, as well as some of the bacteria themselves are washed away by melting ice water. The point that has to be settled is the relative importance of these factors under normal conditions. There is no point in taking excessive care not to lose any of the soluble flavour constituents if the same procedure encourages the development of off-odours and disagreeable flavours.

►WE ASKED THE FISH

With these and similar thoughts in mind, we carried out some tests, the results of which will be of interest to some of our readers. In short, instead of discussing the pros and cons of the argument, we asked the fish. This is how it was done. Seventy 2-day old marker cod were filleted at the local fish plant. This gave us 140 fillets. One half of these were individually wrapped in white onionskin paper and these were wrapped again in waxed wrapping paper. These double-wrapped fillets were placed ten at a time in watertight aluminum containers. To ensure rapid initial chilling, these tins were left for 30 minutes with their lids open in a cool room at 10°F. At this time the fish had cooled to 32°F. but had not frozen. The lids were placed on the cans and they were buried in chipped ice.

The other 70 fillets were placed in groups of five, in 14 clean wooden boxes. These boxes also contained a layer of chipped ice above and below the fillets. The boxes were constructed so that they were not watertight — melting ice water could trickle into the box from outside

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and could also trickle out of the box at the bottom. These were also buried in ice. After 3 days, ten fillets from each lot were taken out for examination and this was repeated daily for 7 days. Examination of the fillets consisted in observing their odour, colour, and texture. Then on each fillet, TMA values* were determined. Also careful records were kept of the temperature of the fillets at the time the boxes were first opened for examination.

►WHAT THE FISH TOLD US

The temperature of the fillets remained between 30 and 32°F. throughout the storage period. As can be seen in the first two columns of the accompanying tables, there was a very obvious difference in the spoilage rate of these two lots of fillets as judged by the appearance, off-odours, and TMA values. After the fifth day, there was no doubt in the minds of those examining the fish, which group they would prefer to cook for dinner. There was no denying the fact that the fish that were protected against the leaching out of soluble nutrients and certain valuable flavour constituents had also gained some very offensive odours.

While the experiment described above was first being considered, curiosity prompted us to ask the fish another question. It was this: "Will fish muscle spoil faster while it is still in the round fish than when it is cut out and stored as a fillet?"

Theoretically this is a difficult question to answer because there are so many other factors that affect the spoilage rate. All other things being equal, the spoilage rate of the dressed or gutted fish will be determined by the extent of contamination in the gut cavity and on the outer surface of the fish. Similarly, other things being equal, the spoilage rate of the fillet depends chiefly upon the numbers and types of bacteria that are picked up by the fillet during its cutting and skinning. Therefore the muscle in a very heavily contaminated, gutted fish is likely to spoil much faster than the fillet from the same fish if it has been cut and skinned under conditions that permit only a very small bacterial pick-up

►ROUND FISH vs. FILLET

So much for theory and generalization! In this particular case we started out with good quality, 2-day old gutted cod that did not have excessive contamination in the gut or on the surface. Also, we were able to cut fillets from these fish under conditions that did not produce excessive contamination on the fillets. They were cut under the normal conditions at the fish plant, by commercial cutters whose only instructions were to use reasonable care. The counts from such fillets varied from 34,000 to 370,000 bacteria per gram with a mean of approximately 98,000.

At the same time that the fillets described above were being buried in ice, 70 of these gutted cod, similar to those from which the fillets had been cut, were also buried in chipped ice. After 3 days 10 fish were removed from the ice and filleted. Each succeeding day 10 more fish were removed and filleted. These fillets were examined and tested in the same way and at the same time as the wrapped and unwrapped fillets that had been buried in ice.

The results are shown in the last columns of Tables I and II. By examining both the description given of the quality of the stored fillets and their TMA values it can be seen that the fillets "stored" in the whole fish were quite similar to those that were wrapped and stored in waterproof containers. The only difference was that they did not go quite so bad during the last two days.

The obvious question to ask is: "If the melting ice water helped to prevent the build-up of spoilage products in the unwrapped fillets, why did it not do the same for the whole fish that were buried in ice?" The most reasonable explanation would be that the spoilage in the whole fish takes place most rapidly in the gut cavity where the slowly running ice water cannot get at it. It is for this same reason that we occasionally hear about the limited success of tests with preservative ices used on gutted fish. Unless special care is taken in placing the ice and fish in the hold, the water from the preservative ice may fail to reach the

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"TMA value" is the number of milligrams of trimethylamine nitrogen that have accumulated per 100 *grams of fish muscle.*

TABLE I. Comments on the colour, texture, and odour of the the wrapped and unwrapped fillets during a storage period of 3 to 9 days at 32°F.

Storage period (days)	Unwrapped fillets in ice	Wrapped fillets in containers	Fillets that had been stored as gutted fish
3	No odours, flesh firm	Slight seaweedy odours: firm but very slightly darker	Slight seaweedy odour
4	No odours, flesh firm	Slight seaweedy, or very slightly sour	Firm and fresh
5	No odour or very slightly seaweedy	Slightly sour	No odour or slightly seaweedy
6	No odour or slightly seaweedy	Slightly sour or musty	Seaweedy
7	No odour	From sour to stale; getting softer	Slightly sour or stale, or strong seaweedy
8	No odour or very slightly sour	Sour — strong sour — putrid, soft	Mostly sour and softer
9	Not much odour, or slightly sour	Sour to putrid — soft and slightly off-colour	Sour — bad — soft

locations where the most serious spoilage is taking place.

In these particular sets of tests it was observed that the fillets remained perfectly edible and reasonably fresh for the following periods:

Unwrapped in ice ..... 8 or 9 days
Wrapped and stored in metal containers ..... 5 or 6 days
Stored as gutted fish ... 6 or 7 days

This, of course, cannot be interpreted as proving the case for the retail merchant who swears by the use of melting ice water. The initial condition of the fish, the rate at which the ice melts, the efficiency and practicability of the handling methods must all be taken into consideration. The experiment has also been one-sided in that we have been more concerned with the offensive products that developed and less concerned with inoffensive and perhaps very useful products that were leached out. But it did show that he had a point to make. Personally, I would much prefer to eat the unwrapped to the wrapped fillets, *after they had been stored 5 or 6 days.* If the sole object was to see how long the fish could be kept in an edible state, the preference would go to chilling by ice water. On the other hand, perhaps you would much prefer the flavour of the wrapped to the unwrapped fillets during the first 4 or 5 days. In actual practice this 4 or 5 days could be easily extended by the use of a suitable antibiotic or some other acceptable preservative.

At the present time, both dry cool storage and ice-water chilling, and various combinations of these, are being used throughout the country with reasonable success in many retail fish stores. It is well, however, to know and to understand both the advantages and the limitations of each method.

These experiments were carried out from the viewpoint of the man who has to sell the fillets. We wanted to know chiefly how these treatments would affect the nose-appeal and the eye-appeal of the customer buying the fish.

Next time we will examine this question from the standpoint of the consumer who eats the fish by trying to determine what differences occur in the taste appeal.

Wrapping of frozen vs. wrapping of fresh fillets

The packaging and storage of frozen fish presents an entirely different problem to that of fresh fish. Every effort is made to keep the frozen product well wrapped and, if possible, in airtight and moisture-proof containers. Whole fish are covered by glazing them in a layer of thin ice; fillets are packed in waxed containers or in cellophane-like wrappers. Such treatment adds significantly to the keeping time of fish and together with the use of low temperature storage facilities, makes possible our present extensive frozen fish trade.

When frozen fish deteriorates there are three principal changes that can take place: it may lose excessive moisture resulting in "freezer burn", the proteins may become denatured giving the fish a tough, rubbery texture; the fat can be oxidized giving the fish mildly unpleasant flavours. Airtight wrapping helps to counteract oxidation and loss of moisture

TABLE II. The average, minimum, and maximum TMA values from 10 unwrapped and 10 wrapped fillets examined each day during storage period of 3 to 9 days at 32°F.

STORAGE PERIOD	UNWRAPPED FILLETS TMA values			WRAPPED FILLETS TMA values			STORED IN THE ROUND TMA values		
	Av.	Min.	Max.	Av.	Min.	Max.	Av.	Min.	Max.
(days)									
3	0.15	0.02	0.30	0.38	0.16	1.05	0.52	0.30	0.84
4	0.16	0.01	0.20	0.78	0.20	3.50	0.70	0.28	2.88
5	0.32	0.02	0.54	0.76	0.52	1.12	0.81	0.40	1.80
6	0.35	0.02	0.80	1.58	0.56	2.90	1.25	0.56	4.16
7	0.27	0.14	0.48	3.71	0.32	14.40	2.32	0.88	3.88
8	0.43	0.14	0.98	5.71	2.20	10.70	2.95	0.68	4.60
9	1.09	0.48	4.40	9.90	4.80	17.60	5.56	2.54	9.10

and generally tends to retard deterioration in frozen fish muscle.

It is important to realize that in the spoilage of frozen fish there is little or no production of offensive volatile substances that could accumulate within the package. It is interesting to contrast this with what occurs when unfrozen fish begins to spoil.

Instead of the relatively slow and comparatively limited changes that take place in the frozen fish, the bacteria on the unfrozen fish rapidly bring about extensive breakdown of the proteins, fats, and other components of the fish muscle. A whole host of by-products are formed. Among these are many volatile substances that we recognize as the off-odours that develop as the fish spoils. The first ones to be formed are often relatively inoffensive, but as spoilage continues, they become more and more obnoxious; finally the combination of many of these gases results in what we recognize as putrid fish.

If unfrozen fish is enclosed in an airtight container, these offensive gases accumulate. In many instances, their presence may actually speed up further deterioration. If the fish are exposed to the air or better still, if they are held in ice and are being slowly washed with melting ice-water, these gases escape into the atmosphere or are washed away.

There is another point that must be taken into consideration. Some bacteria act quite differently in the presence or in the absence of air. When atmospheric oxygen is available, they decompose the fish in one way; in the absence of air they decompose it in another. way. Unfortunately, in the absence of air the most offensive spoilage odours are produced.

This can be summed up in two simple statements:

1) Airtight wrapping retards the start of spoilage in frozen fish, and once spoilage has commenced, it does not make it any worse.

2) Airtight wrapping does not retard spoilage in unfrozen fish, and once spoilage has commenced, it accumulates the offensive gases and in many instances it actually speeds up the spoilage process.

To wrap or not to wrap?

This does not mean that fresh fillets should never be wrapped in cellophane or other airtight containers. There are some very distinct advantages to such wrappers, particularly if they are transparent. They allow the customer to see the fish she is buying and at the same time protect the fish from direct contact with human hands. No one likes to buy a raw food product, such as fish, that has been previously handled by other people.

What then are we to conclude from these experiments where we have asked questions of the fish itself, and from this brief discussion of the difference in the spoilage of fresh and frozen fish?

► 1) Do not rely on wrapping to retard the spoilage of unfrozen fillets.

► 2) Do not wrap fillets that are not of good quality to begin with, or fillets that may be stored for periods of time that are approaching their normal shelf life.

►3) *Once a wrapped fillet begins to spoil,* offensive odours accumulate within the wrapper and further deterioration is generally accelerated.

►4) Generally a wrapped fillet present a more acceptable appearance than an unwrapped fillet. It keeps human hands from coming in direct contact with the fish and it tends to support and hold together fillets that might otherwise flake open or break into sections.

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BSERVATIONS ON THE FUNCTION OF SODIUM IN THE METABOLISM OF A MARINE BACTERIUM

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(Received for publication, January 16, 1958)

Bacteria have been found in the sea which are distinguishable from land fresh water forms by having a highly specific and readily demonstrable uirement for Na^+ for growth (1). Like other bacteria investigated, y also require K^+. When Na^+ and K^+ were present in a medium at the centrations of these ions in sea water, the concentration of Na^+ was nd to be less and K^+ greater in the cells than in the medium. Further- re, the quantitative requirements of the marine bacteria for Na^+ and proved to be very similar to those of animal cells in tissue culture. rine bacterial cells and animal cells are thus very similar with regard to ir relation to their inorganic environment, a point which may be of siderable significance from the standpoint of evolution.

Both Na^+ and K^+ have been found to be essential for the oxidation of genous substrates by cell suspensions of a marine bacterium (2). A re detailed investigation has shown that the requirement for Na^+, but for K^+, varied according to the substrate being oxidized. Individual ymes tested in cell-free extracts of the organism, on the other hand, did require the addition of Na^+ for activity.

EXPERIMENTAL

The organism used was isolated from sea water and has been tentatively ntified as a species of either a *Pseudomonas* or a *Spirillum*. The pro- ures for growing the organism and preparing cell suspensions have n described, and the medium used was only a slight modification of one employed previously (2). To obtain cell suspensions capable of dizing malate, citrate, galactose, and glucose, cells had to be suitably pted by incorporating the substrate to be oxidized into the growth dium. For this purpose the substrate was added at a 0.5 per cent level. ls adapted to the oxidation of galactose could also oxidize glucose and e used for the glucose oxidation study. The cells were washed and pended in a 0.052 M $MgCl_2$ solution for the purposes of this investigation. dation of substrates by whole cell suspensions was followed mano- rically by using a Warburg respirometer.

533

Cell-free extracts of the organisms were prepared by sonic disruptio the cells in a Raytheon 10 kc. sonic oscillator. Succinic dehydrogenase assayed manometrically in the extract by measuring the rate of oxy uptake in the presence of methylene blue (3). KCN was omitted w the effect of Na^+ and K^+ on the enzyme system was being determin Fumarase was measured spectrophotometrically by observing the incre in optical density at 240 mμ with malate as substrate (4). Malic de drogenase activity was determined by following the rate of reduction TPN (triphosphopyridine nucleotide) spectrophotometrically at 340 (*cf.* (5)). For the cell-free extract studies tris(hydroxymethyl) ami methane buffers were used throughout. Protein was estimated by biuret method (6) with crystalline pepsin (Worthington Biochemical C poration) as a standard.

Na^+ and K^+ determinations were carried out with the aid of a fla photometer attachment for a Beckman DU spectrophotometer equip with a photomultiplier.

Results

With either acetate, butyrate, or propionate as substrate in the Warb flask, a 50 mM solution of Na^+ was required for maximal rate of oxidati

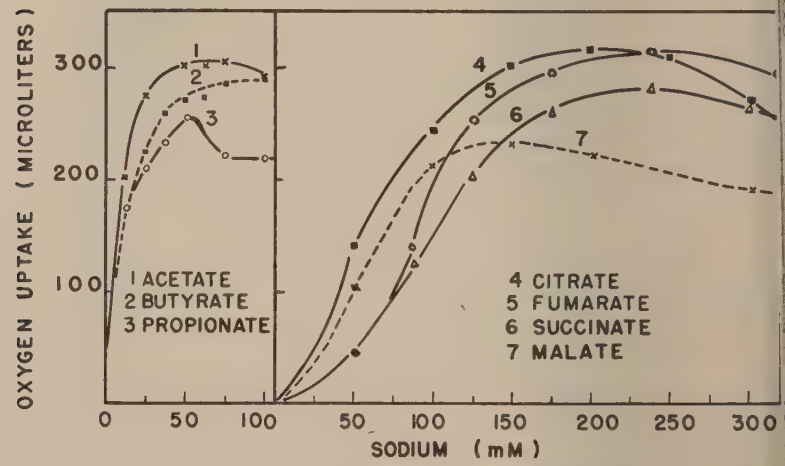

FIG. 1. Na^+ requirements for oxidation of various substrates by whole cell su pensions of a marine bacterium. Each Warburg vessel contained PO_4 buffer (K 46 mM, pH 6.7, KCl 80 mM, $MgCl_2$ 3.4 mM, substrate (K^+ salt) 3.57 mM, 7 mg. d weight of cells; total volume 2.8 ml.; center well 0.2 ml. of 20 per cent KOH; g phase air; temperature 25°. Values recorded corrected for endogenous oxygen u take at each level of Na^+ tested and represent oxygen uptake after the followi incubation periods: fumarate 60 minutes, succinate and citrate 70 minutes, acetat propionate, and butyrate 75 minutes, malate 90 minutes.

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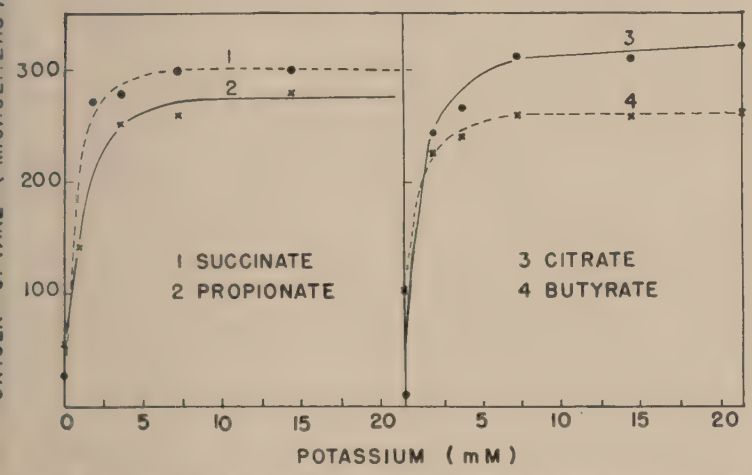
the cell suspension (Fig. 1). For the oxidation of malate about three es and for that of citrate, fumarate, and succinate about four times, level of Na^+ was required. When the requirements for K^+ for oxida- were determined, no variation with substrate could be detected (Fig. The Na^+ and K^+ requirements for the oxidation of glucose and ctose by whole cell suspensions were determined and found to be almost tical with those found optimum for the monobasic acids.



IG. 2. K^+ requirements for oxidation of various substrates by whole cell suspen- of a marine bacterium. Each Warburg vessel contained tris(hydroxymethyl)- nomethane buffer, adjusted to pH 6.7 with H_3PO_4, 46 mM, NaCl 77 mM for mono- c acids and 200 mM for di- and tribasic acids, $MgCl_2$ 3.4 mM, substrate (Na^+ 3.57 mM, 7 mg. dry weight of cells; total volume 2.8 ml.; center well 0.2 ml. of er cent KOH; gas phase air; temperature 25°. Values recorded corrected for genous oxygen uptake at each level of K^+ tested and represent oxygen uptake r the following incubation periods: citrate 55 minutes, succinate 75 minutes, rrate 90 minutes, propionate 150 minutes.

Extracts of the organism were prepared by sonic disruption of the cells. imal conditions for the determination of the activity of succinic de- rogenase, fumarase, and malic dehydrogenase in the extract were blished. The relative activity of each enzyme in the presence of ious concentrations of Na^+ and K^+ was then determined (Table I). h succinic dehydrogenase and fumarase were found to be most active n neither Na^+ nor K^+ was added to the medium. In contrast to the vity of the other two enzyme systems studied, that of malic dehy- genase was negligible unless K^+ was present. A 167 mM concentration K^+ was found to produce an optimal response. In all cases, a level of

Na^+ and K^+ which would promote maximal rate of oxidation of the s
strates by whole cell suspensions (200 mM Na^+ and 33 mM K^+) depres
enzyme activity in the cell-free extracts. Various other concentrati
of Na^+ and K^+ tested had no additional stimulatory effect. Analyses
contaminating traces of the ions in those reaction systems which contai
no added Na^+ and K^+ revealed that for the succinic dehydrogenase syst
the Na^+ and K^+ levels were 1.2 and 1.8 mM, for fumarase 0.02 and 0.0
mM, and for malic dehydrogenase 0.26 and 0.18 mM, respectively.

The results recorded in Table I for succinic dehydrogenase were

TABLE I

Activity of Succinic Dehydrogenase, Fumarase, and Malic Dehydrogenase in Cell-Free Extracts of Marine Bacterium at Various Concentrations of Na^+ and K^+

Ions added		Succinic dehydrogenase	Fumarase	Malic dehydrogena
Na^+	K^+	Relative activity*		
mM	*mM*	*per cent*	*per cent*	*per cent*
0	0	100	100	8
200	33	65	25	37
200		55	36	5
33		88	97	8
	33	70	77	82
	200†	68	38	100
33	200†	58	30	100

* For each enzyme the maximal activity attained was assigned the value of 1
The activity under other conditions is expressed as per cent of the maximal ac
vity.

† For malic dehydrogenase the level of K^+ for the optimal rate of oxidati
(167 mM) was used.

tained in a system containing no KCN to avoid adding K^+. With KC
present, however, the enzyme did not respond to added Na^+.

DISCUSSION

The data presented reveal a quantitative requirement for Na^+ for t
oxidation of substrates by whole cells which varied with the substra
tested, while the individual enzymes present in extracts of the cells showe
no dependence on the presence of Na^+, at least in any appreciable amount
for activity. To account for the large difference in Na^+ requirement f
oxidation by whole cells of the monobasic acids and hexose sugars o
the one hand and for the di- and tribasic acids on the other, one mu
conclude either that the two groups of compounds are being oxidized b

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erent oxidative pathways or that Na^+ is involved in the transport of least some of the substrates across the cell membrane.

Considerable evidence that a tricarboxylic acid cycle exists in this or- ism has been obtained. Except for certain unusual features about oxidation of propionate (7), there is no evidence to suggest that the nobasic acids are oxidized by other than this pathway. Since this abolic pathway is cyclic in nature, a requirement for Na^+ at any step for the transport of electrons to oxygen could not account for the dif- nces in the Na^+ requirement of whole cells.

By the application of the nuclear spin resonance technique, evidence has n obtained indicating that Na^+ forms complexes with common metabo- s (8). The strongest interactions with Na^+ have been found to occur ong compounds forming chelates. The monobasic acids concerned e show little tendency to form chelates, while the di- and tribasic acids form metal complexes (9). Differences in the Na^+ requirement for lation of the two groups of compounds can then best be accounted for assuming that each of the di- and tribasic acids must form a complex n Na^+ before being transported across the cell membrane. The Na^+ uirement for the oxidation of the monobasic acids and monosaccharides uld then be a reflection of the amount of Na^+ required intracellularly er for oxidative metabolism or to operate the Na^+ pump (10), while additional amount needed for the oxidation of the di- and tribasic ds would represent the Na^+ required to form the various complexes. h an explanation would also serve to account for Na^+ transport into cell when appropriate substrates are present in the medium, but not n compounds which would not form associations with Na^+ are being lized.

ssays for the activity of many tricarboxylic acid cycle enzymes in nal tissue homogenates are carried out in the absence of added Na^+ K^+ (11). Animal cells in tissue culture have also been shown to require and K^+ for growth (12). It remains to be seen whether the phenome- described here for marine bacterial cells occurs in animal tissues as well. having a requirement for K^+, the malic dehydrogenase of this organism mbles the malic enzymes of *Lactobacillus arabinosus* (13) and *Moraxella ffi* (14). It differs from them in being TPN-specific rather than di- sphopyridine nucleotide-specific and in the fact that the reaction malate as substrate can be reversed by adding oxalacetate.

SUMMARY

With either acetate, butyrate, propionate, glucose, or galactose as strate, cell suspensions of a marine bacterium required an approximately mM concentration of Na^+ for maximal rate of oxidation. For the oxida-

tion of malate three times and for citrate, fumarate, and succinate abou four times, this level of Na^+ was required. No variation in the K^+ re quirement with different substrates could be detected.

In cell-free extracts of the organism, succinic dehydrogenase and fu marase were more active in the absence of added Na^+ and K^+ than in th presence of these ions. Malic dehydrogenase required the addition of K for maximal activity. In all cases, levels of Na^+ and K^+ required fo maximal rate of oxidation by cell suspensions inhibited the activity o the enzymes in the cell-free extracts.

It was concluded that the differences in the Na^+ requirement of ce suspensions with substrate observed could best be accounted for by as suming that the di- and tribasic acids, but not the monobasic acids an hexose sugars form complexes with Na^+ before crossing the cell membrane

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RIBONUCLEASE ACTIVITY OF FISH MUSCLE EXTRACTS[1]

NEIL TOMLINSON

Abstract

A ribonuclease was partially purified from muscle of lingcod (*Ophiodon elongatus*). The preparation degraded yeast and lingcod ribonucleic acid. Four 3′-mononucleotides were identified as digestion products. The enzyme was readily destroyed by heat and acid (10 minutes at 58° C., pH 2.5), and was inhibited strongly by Zn^{++}, Cu^{++}, monoiodoacetate, formaldehyde, and NaF. Its pH optimum was 6.5. Ribonuclease activity was also demonstrated in muscle extracts from spring salmon, lemon sole, dogfish, sculpin, herring, and swordfish. The muscle extracts of all fish examined possessed phosphodiesterase activity. The ribonuclease in lingcod extract could be separated from two phosphodiesterases also present by zone electrophoresis.

Introduction

The work of Tarr (1) indicated the presence in lingcod (*Ophiodon elongatus*) and rock cod (*Sebastodes* sp.) muscle of enzymes capable of degrading yeast ribonucleic acid (RNA), ribonucleotides, and ribonucleosides with the release of free ribose. Since then he has made a detailed study of several of these enzymes, in particular those concerned with the metabolism of nucleosides (2,3). While these studies have elucidated certain of the later enzymic steps in the breakdown of RNA in fish muscle, details of the earlier steps, leading presumably to the formation of nucleosides, have remained obscure. The present work was undertaken with the object of investigating fish muscle extracts for ribonuclease activity.

Materials and Methods

Fish from which extracts were to be prepared were obtained fresh. The fish were either used at once, or were frozen until used. Experience has shown that fish may be held frozen for months and still yield enzymatically active extracts.

Protein was measured by the method of Snow (4) or by the method of Lowry *et al.* (5).

Orthophosphate was measured by the method of Lowry and Lopez (6) as modified by Bruemmer and O'Dell (7). Total phosphorus was determined by the method of King (8).

Yeast RNA (Schwarz Laboratories) for use as substrate was purified by precipitation with alcohol from cold aqueous solution at pH 4.3, and finally obtained as the sodium salt (8.1% phosphorus) by precipitation from cold, neutral solution (9).

All absorption measurements were made with a Beckman model DU spectrophotometer, 1 cm. cells being used throughout. Ribonuclease (RNase) activity was measured by a modification of the spectrophotometric method of McDonald (10). One unit of RNase activity was defined as that activity

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which, in 30 minutes, released from RNA material soluble in a solution prepared as described below, sufficient to provide an optical density of 0.050 as measured at a wavelength of 260 mμ. A digestion mixture, consisting of 0.5 ml. sodium succinate buffer, 0.1 *M*, pH 6.0, 0.5 ml. sodium ribonucleate solution (380 μg. P/ml.), and 1.0 ml. of enzyme solution, was incubated at 25° C. Aliquots (0.5 ml.) of the mixture were removed immediately after the addition of the enzyme and after a 30-minute incubation period. These aliquots were at once added to an equal volume of MacFadyen's uranyl acetate trichloroacetic acid (UTCA) reagent (11). After being kept $\frac{1}{2}$ hour at room temperature, the mixtures were filtered and 0.4 ml. of the filtrates were diluted to 5 ml. with distilled water. The optical densities ($\log_{10} I_0/I$) of the resulting solutions were determined at 260 mμ, the reference blank being 0.2 ml. of UTCA reagent diluted to 5 ml. with water. Digestion blanks with substrate or with enzyme omitted were employed. The units of activity in the digestion mixture (and hence in 1 ml. of enzyme extract) were determined by reference to a standard curve—optical density vs. activity units—prepared from data obtained by measuring the activity of a series of dilutions of a single enzyme extract. The activity unit defined above is equivalent to the release of 4 μg. of phosphorus soluble in the UTCA reagent per milliliter of digestion mixture per half hour. It is thus approximately equal to the RNase activity unit of McDonald (10). In the measurement of RNase in extracts following electrophoresis on paper, because of the low activities to be measured, longer incubation periods were necessary (6 to 9 hours), and results are reported simply as optical density of the UTCA solutions at 260 mμ.

Phosphodiesterase activity was measured with *bis*(*p*-nitrophenyl)phosphate as substrate. The digestion mixture consisted of sodium succinate buffer pH 6.0, 0.025 *M*, *bis*(*p*-nitrophenyl)phosphate (sodium salt) 0.0018 *M*, and enzyme. The incubation temperature was 25° C. Aliquots (0.33 ml.) of the reaction mixture were removed immediately after addition of the enzyme and after a suitable period of incubation (normally 2 hours). These were at once added to 3 ml. of 0.1 *N* NaOH. The optical densities of the resulting solutions were measured at 400 mμ and the *p*-nitrophenol released determined by reference to a suitable calibration curve. Reaction blanks with enzyme omitted were used.

Phosphomonoesterase activity was measured either by determination of orthophosphate (vide supra) released from RNA as substrate (reaction mixture deproteinized with an equal volume of ice-cold 10% TCA) or by measurement of *p*-nitrophenol released from disodium *p*-nitrophenyl phosphate (Sigma Chemical) as substrate. The reaction mixture with the latter compound as substrate consisted of sodium succinate buffer, pH 4.5, 0.025 *M*, substrate, 0.018 *M*, and enzyme. The incubation temperature and time were 25° C. and $\frac{1}{2}$ hour, respectively. Measurements were performed as for the phosphodiesterase determinations described above.

When enzymic reactions were allowed to proceed for longer periods than a few hours, chlorotetracycline.HCl (5 p.p.m.) was added to the reaction mixture to prevent bacterial growth. At the end of the experiment these

reaction mixtures were examined microscopically for bacteria by means of direct smears, results being discarded if any evidence of bacterial growth was found.

Electrophoresis of proteins was carried out using Whatman No. 3 paper at 1° C. and a field strength of 25 v./cm. for 3½ hours. The apparatus used was manufactured by the E–C Apparatus Company, Swarthmore, Pa. The paper sheets were clamped in the apparatus between teflon sheets during the electrophoretic run. The use of field strengths lower than 25 v./cm. was found to be unsatisfactory, as the much longer time required resulted in poor separation due to diffusion. The buffer solution found most suitable, of several tried for this purpose, was sodium cacodylate, μ 0.05, pH 7.0. Protein on papers was located, following electrophoresis, by staining with bromophenol blue by Kunkel and Tiselius' modification (12) of the method of Durrum (13). The bound stain was estimated by the method of Kunkel and Tiselius (12). Segments 2 mm. wide were cut from the paper, eluted 30 minutes in 3 ml. of 0.01 *N* NaOH, and the optical density of the eluates measured at 575 mμ.

For assay of enzymes separated by paper electrophoresis, segments cut from the papers without drying were added directly to suitable buffer–substrate solutions. Blanks in such assays consisted of paper segments cut from protein-free areas of the paper, then treated in the same manner as the enzyme-containing segments.

Electrophoresis of nucleotides was conducted under conditions similar to those used in the electrophoresis of proteins, but with buffer solutions recommended by Crestfield and Allen (14). These were (I) ammonium formate, μ 0.1, pH 3.5, (II) sodium phosphate, μ 0.1, pH 5.8, and (III) sodium phosphate, μ 0.1, pH 6.2.

Paper chromatography of nucleotides was carried out on Whatman No. 1 or No. 3 paper with the following solvent systems: (A) isobutyric acid – 0.5 *N* ammonium hydroxide (10/6, v/v), pH 3.7 (15); (B) 0.1 *M* sodium phosphate pH 6.8 – ammonium sulphate – isopropanol (100/60/2, ml./g./ml.) (16); (C) 1 *M* ammonium acetate pH 9.0 – ethanol (30/70, v/v) saturated with sodium tetraborate (17).

Nucleotides were located visually on paper with the aid of a Mineralight lamp. The nucleotides were estimated by elution in 4 ml. of 0.01 *M* HCl followed by measurement of the optical density of the eluates at the appropriate wavelength in the ultraviolet region (18). Reference blanks were prepared from pieces of paper of the same sizes as those of the pieces containing the nucleotides, but cut from nucleotide-free portions of the paper at the same distances from the origin.

Results

An extract with RNase activity was prepared from lingcod as follows, all procedures being conducted at 5° C. or lower unless indicated otherwise. Muscle tissue was blended with twice its weight of demineralized water. The resulting suspension was adjusted to pH 6.2, and the insoluble material

removed by centrifuging the suspension 45 minutes at 2500 *g*. The supernatant solution was adjusted to pH 7.0 and solid ammonium sulphate was added to 80% saturation, the pH being maintained at 7.0 by the addition of 5 *N* NaOH as needed. The solution was allowed to stand 30 minutes, after which the precipitated protein was removed by centrifugation as above. The supernatant solution was discarded and the precipitate was transferred to a dialysis bag with the aid of a few milliliters of distilled water and then dialyzed for 20 hours against several changes of distilled water. The contents of the dialysis bag were adjusted to pH 6.8 and centrifuged for 15 minutes at 25,000 *g*. The precipitate was discarded and the supernatant solution concentrated to a small volume by lyophilization. The pH of the concentrated extract was next adjusted to pH 4.5 (1 *N* HCl) and the solution held 1 hour at 25° C. Precipitated protein was removed and discarded, the pH of the solution was brought to 9.0 (1 *N* NaOH), and the solution held at 58° C. for 10 minutes. The precipitate was removed and discarded and the pH of the solution adjusted to 6.8. Extracts prepared in this manner have been held for several days at 1° C. or for months at −20° C. with very little loss in activity. Table I summarizes the results of such a procedure.

TABLE I

PARTIAL PURIFICATION OF RIBONUCLEASE FROM MUSCLE OF LINGCOD (*Ophiodon elongatus*)

Extract following	Total activity*	Ribonuclease		Phosphodiesterase		Phosphomonoesterase	
		Specific activity†	% recovery	Specific activity‡	% recovery	Specific activity§	% recovery
Clarification	2952	1.64	100				
$(NH_4)_2SO_4$ precipitation, lyophilization, and isoelectric precipitation	1260	11.5	43	0.19	100	7.5	100
10 min. at 58°, pH 9.0	638	7.0	22	0.14	62	0	0

*Units from 800 g. lingcod muscle.
†Units/mg. protein N.
‡μM. *p*-nitrophenol released/2 hr./mg. protein N.
§μM. *p*-nitrophenol released/hr./mg. protein N.

Attempts to obtain fractionation of extracts by ammonium sulphate precipitation were unsuccessful, but precipitation at 80% saturation did provide some concentration of the RNase activity. Isoelectric precipitation at pH 4.5 effected a small increase in the specific activity of the extract and heating at pH 9.0 eliminated all traces of phosphomonoesterase activity with either RNA or *p*-nitrophenyl phosphate as substrates under the test conditions described above. The extract does, however, still contain a Zn-activated phosphomonoesterase at this stage. The RNase activity was found to be much more sensitive to heating at pH 2.5 or 3.5 than at pH 6.6 or 9.0. At these pH's 99%, 85%, 21%, and 50%, respectively, of the total activity was destroyed in 10 minutes at 58° C.

The partially purified extract has phosphodiesterase activity as measured with *bis*(*p*-nitrophenyl)phosphate as substrate (Table I). However, the data indicate that the RNase and phosphodiesterase activities are not due to the

same enzyme, as the losses in the two activities and also the changes in their specific activities during the pH 9.0 heating step are not equal. It should be noted that the loss in phosphodiesterase activity indicated is a maximum, as a portion of the loss must be attributed to the removal of the phosphomonoesterase which, when present, increases the apparent activity of the phosphodiesterase under the test conditions.

An extract, freed from phosphomonoesterase activity as described above, was subjected to electrophoresis on paper with the results recorded in Fig. 1. The position of the RNase activity did not correspond to the position of the two major phosphodiesterase activities, and the position of none of these three activities corresponded exactly with either of the two major protein peaks. A portion of each phosphodiesterase-containing area was obtained free from RNase activity, consequently it appears that neither of the diesterases can degrade RNA. On the other hand, the RNase may have low activity against *bis*(*p*-nitrophenyl)phosphate, as eluting the peak of the RNase activity (1 cm. segment) from the paper and subjecting it to electrophoresis once more did not greatly reduce the phosphodiesterase activity found at

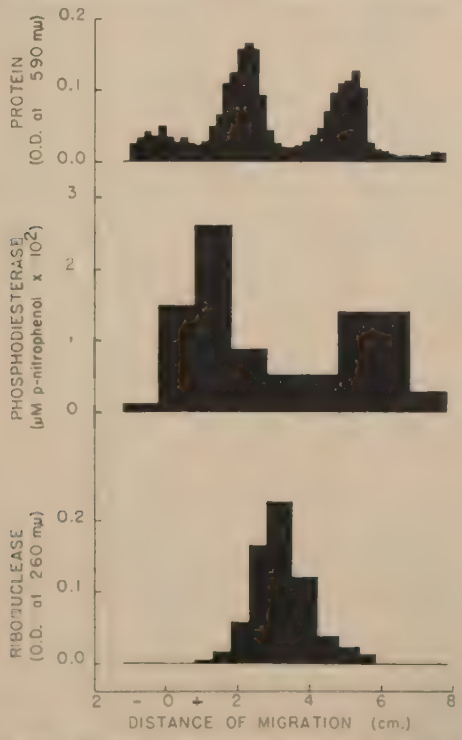

FIG. 1. Electrophoresis of lingcod muscle extract on paper; 9.6 μg. protein N applied cm. length of starting line. Total protein N in strip for protein-bound dye determination, 14 μg., for phosphodiesterase, 115 μg., and for ribonuclease, 48 μg. *p*-Nitrophenol released by phosphodiesterase control, 0.0825 μM. Increase in O.D. at 260 mμ by RNase control, 0.633.

the position of the RNase peak. However, the phosphodiesterase activity found in this position is of such a low order of magnitude that the accuracy of the measurement is perhaps open to question.

Attempts to chromatograph an enzyme extract on celite by the method of Martin and Porter (19) and on XE-64 resin by the method of Hirs *et al.* (20) were unsuccessful. Failure with the first method may have been due to the low specific activity of the enzyme solution together with inhibition of the enzymes by the eluting solution. Failure with the second method was due to irreversible binding of protein to the resin at the low pH necessary to obtain uptake (21).

The influence of pH on the RNase and phosphodiesterase activities of the preparation was investigated, and each was found to exhibit a maximum activity near pH 6.5 (Fig. 2). Only one activity peak was found in the curve for phosphodiesterase, although two enzymes were present.

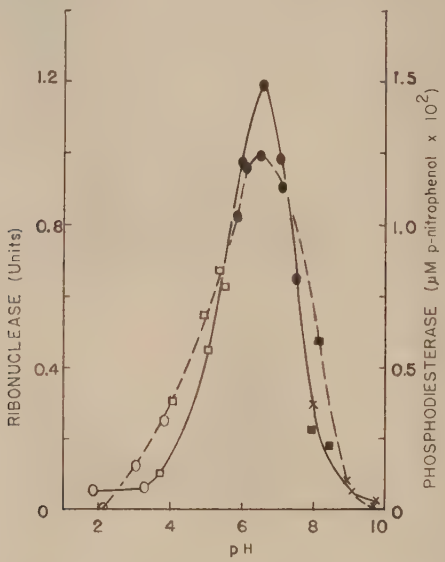

FIG. 2. Influence of pH on the RNase and phosphodiesterase activity of lingcod muscle extract.
RNase ————. Phosphodiesterase — — — . Buffers (0.025 *M*): glycine.HCl ○————○, sodium acetate □————□, sodium cacodylate ●————●, sodium diethylbarbiturate ■————■, sodium borate ×————×. The pH's indicated are those of the complete reaction mixture.

The effect of a number of possible inhibitors or activators on the RNase and phosphodiesterase activities were examined; 0.05 *M* NaCl, 0.005 *M* Na_2SO_4, 0.0005 *M* NaF, 0.005 *M* $MgSO_4$, and 0.005 *M* $CaCl_2$ had little or no effect on either activity; 0.005 *M* $ZnSO_4$, 0.005 *M* $CuSO_4$, 0.005 *M* NaF, and 0.1 *M* NaF inhibited both activities; RNase to the extent of 59, 93, 33, and 89% respectively, and phosphodiesterase to the extent of 69, 93, 21, and 98%. Disodium ethylenediamine tetraacetate (EDTA), 0.025 *M*, inhibited

RNase to the extent of 23% but was without effect on phosphodiesterase. At the same concentration EDTA reversed the inhibitory effect of 0.005 *M* $ZnSO_4$ on both activities. Formaldehyde, 0.5%, inhibited RNase 88% and phosphodiesterase 21%. Sodium iodoacetate at 0.01, 0.005, and 0.0005 *M* concentrations inhibited RNase 98, 91, and 55%, respectively, whereas no inhibition of phosphodiesterase was detected at these concentrations. In the foregoing, the enzyme extract was incubated in buffered solution with the compound being tested for 15 minutes before the substrate was added.

Fig. 3 shows the course of breakdown of RNA by a lingcod muscle extract without phosphomonoesterase activity removed. The increase in ultraviolet light-absorbing materials soluble in UTCA reagent paralleled very closely the increase in soluble phosphorus, and practically the whole (99%) of the RNA phosphorus was rendered soluble in the UTCA reagent. The course of breakdown of RNA by an extract freed from phosphomonoesterase activity was also examined, and apart from the fact that no orthophosphate appeared, was found to be similar, although the rate of digestion decreased somewhat more rapidly, presumably owing to the accumulation of products of the reaction in the absence of phosphomonoesterase action (10).

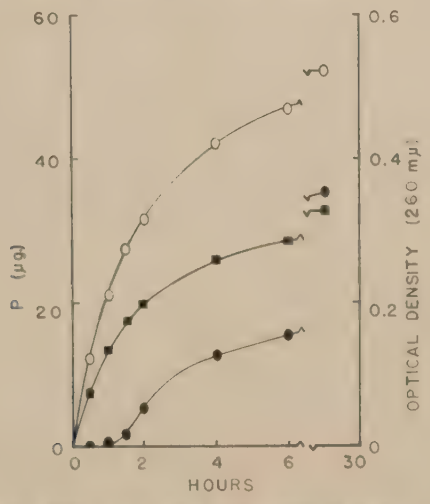

FIG. 3 Digestion of yeast RNA by lingcod muscle extract. Increase in UTCA-soluble P, ○——○; orthophosphate, ●— —●; UTCA-soluble, ultraviolet-absorbing material, ■——■. Total RNA P insoluble in UTCA at start, 52.5 μg.

The ability of these extracts to degrade RNA prepared from lingcod muscle by the method of Volkin and Carter (9, 22) was investigated. This material was degraded, but at a rate only about 15 to 20% of that observed with the yeast RNA preparation. As a matter of interest, the rates of digestion of these two substrates by crystalline pancreatic ribonuclease (Nutritional Biochemicals) was also determined. Again, the yeast preparation was the more rapidly digested of the two.

The final products of the breakdown of RNA by the phosphomonoesterase-free extract were investigated by incubating RNA with the extract for 48 hours, then separating the products of the reaction by ionophoresis in buffer I. From their migration on ionophoresis, and from their ultraviolet absorption spectra in neutral, acid, and alkaline solution (23), adenylic, cytidylic, guanylic, and uridylic acids were tentatively identified as products of the reaction. By chromatography in solvent C, the possibility that any of the four were 5′ isomers was eliminated. The four 5′ mononucleotides have R_f values of 0.02 or less in this solvent, while the 2′ and 3′ isomers of adenylic, cytidylic, guanylic, and uridylic acids have R_f values of 0.1, 0.11, 0.065, and 0.13, respectively. The four mononucleotides from the digestion mixture behaved as did known mixtures of the 2′ and 3′ isomers in this solvent system. Adenylic and guanylic acids from the reaction mixture were finally identified as the 3′ isomers by co-chromatography on paper with known compounds in solvent B. Cytidylic and uridylic acids were identified as the 3′ isomers by ionophoresis. Buffer III was used with cytidylic acid, and buffer II with uridylic acid. These buffers may be used to distinguish between the three isomers of these nucleotides. Under the conditions described above, in 3 hours and 50 minutes in buffer III, the 5′-, 2′-, and 3′-monophosphates of cytidine migrated 21, 22.5, and 23.7 cm., respectively, and in the same time in buffer II, the 5′-, 2′-, and 3′-monophosphates of uridine migrated 19.5, 22, and 23 cm. Examination of the reaction mixture by two-dimensional paper chromatography with solvent A as the first, and solvent B as the second solvent, revealed four ultraviolet-absorbing spots corresponding to the positions of the four mononucleotides identified above, and a small amount of ultraviolet-absorbing material that streaked a short distance in solvent A, but that did not move appreciably in solvent B. Spectrophotometric estimation of the mononucleotides separated from the reaction mixture by ionophoresis resulted in 83% of the RNA phosphorus added to the reaction mixture being accounted for in the four mononucleotides. (RNA phosphorus added 3.92 μM. Recovered in adenylic acid 0.85 μM., cytidylic acid 0.61 μM., guanylic acid 1.08 μM., and uridylic acid 0.71 μM.) The possibility that small amounts of other mononucleotides may have been formed cannot be ruled out, but none were detected by the methods used. A similar procedure to the above was carried out, but with an enzyme solution purified by electrophoresis on paper as described above, elution and concentration by lyophilization, then electrophoresis on paper once more, followed again by elution and concentration. All four 3′-mononucleotides were again identified from the digestion mixture, but only 25% of the RNA phosphorus was accounted for by these compounds. Examination by two-dimensional paper chromatography, as described above, revealed the presence of the four mononucleotides together with a relatively large amount of material streaked a short distance from the origin in solvent A, but not moving appreciably in solvent B. Thus the enzyme purified by repeated electrophoresis releases

all four 3′-mononucleotides from RNA, but the extent to which it can degrade RNA is not clear, for sufficient purified enzyme has not yet been available to carry out what could be considered an exhaustive digestion of the substrate. The electrophoretic method of purification of the enzyme was laborious and yielded only very small amounts of the purified material. Further examination of the initial stages of the breakdown of RNA by this enzyme must await the development of a purification method that will provide a much higher yield.

Extracts were prepared from muscle of red spring salmon (*Oncorhynchus tshawytscha*), lemon sole (*Parophrys vetulus*), dogfish (*Squalus suckleyi*), sculpin (Cabezon *Leptocottus armatus*), herring (*Clupea pallasii*), and swordfish (*Xiphias gladius*). These extracts, after treatment by the ammonium sulphate precipitation, dialysis, and lyophilization steps of the purification procedure, were examined for ability to degrade RNA and *bis*(*p*-nitrophenyl)-phosphate. Each extract contained enzymes capable of attacking both substrates. It thus appears probable that such enzymes are widely distributed in muscles of marine fish.

Discussion

The quantities of RNase activity found in the fish muscle extracts, although on a fresh weight basis very much less than those reported in beef pancreas extracts (24), and in calf spleen and liver extracts (25), would be sufficient under suitable conditions to bring about rapid decomposition of the quantities of ribonucleic acid found in fish muscle (22). The enzyme could play a significant part in the post mortem formation of free ribose in fish muscle, as suggested by Tarr (26).

A comparison of the lingcod RNase activity with RNases from other sources is interesting, and emphasizes the rather wide variety of enzymes now known that degrade RNA.

In its lability to heat at an acid pH, lingcod muscle RNase resembles certain RNases from calf pancreas, spleen, and thymus (27, 28, 29), and the acid RNase from rat liver (30), but differs markedly from other RNases (30, 31, 32, 33, 34) in this respect.

The inhibitory effects of $ZnSO_4$, $CuSO_4$, monoiodoacetate, and formaldehyde on lingcod RNase are similar to the effects of these compounds on pancreatic RNase reported by Zittle (35, 36), but the lingcod RNase is inhibited strongly by high concentrations of NaF that are without effect on pancreatic RNase (37). Also, the sensitivity of the lingcod enzyme to iodoacetate and NaF is in sharp contrast to the insensitivity of RNase T_1 from Takadiastase to these compounds (33, 34).

In view of the partial purification procedure that has been used in the present work, it appears unlikely that the lingcod RNase activity is due to the action of a group of enzymes similar to those from pancreas (29). In addition,

the fact that the lingcod RNase cannot be chromatographed on XE-64 resin further distinguishes it from pancreatic RNase (20, 38), and also certain spleen and liver RNases (32).

The release of all four 3′-mononucleotides from RNA by the enzyme purified by repeated electrophoresis indicates that its action is different from those of the RNases from rye grass (39) and *Asterococcus mycoides* (40) and the phosphodiesterase from snake venom (41), each of which releases nucleoside 5′-phosphates, the RNase from pea leaves that releases cyclic nucleotides (42), and the RNases from calf spleen and liver that release pyrimidine mononucleotides only (32). However, the products indicate its action may be similar to that of Hilmoe and Heppel's spleen nuclease fraction III (28, 43, 44) which it resembles also in pH optimum, apparent lack of activation by metallic ions, and sensitivity to heat.

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Some Principles Involved in Regulation of Fisheries by Quota

by

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All kinds of fishery regulations are designed to stop people from catching fish, even when they promise larger catches later on as a reward. Regulations may operate in one of a number of ways: 1. They may reduce the catch of a species at all sizes, or only at some particular range of sizes; 2. They may prohibit fishing in certain places; 3. They may prohibit fishing at certain times of year, or certain times of the day; 4. They may prohibit the use of certain kinds of gear; 5. They may prohibit taking fish in excess of a certain number (or weight) by any one individual, or boat; 6. They may prohibit taking fish in excess of a given total number or weight, each year, from the stock in question.

The motives which lead to these regulations are all, I believe, economic in the broad sense. However they are classifiable in two groups: those which aim at direct economic ends, and those which aim at increasing the general level of potential catch. Among the more direct economic aims may be, for example: 1. a desire to share the catch among as large a number of fishermen as possible (e.g., bag limits in sport fisheries); 2. a desire to restrict the number of individual fishermen or boats so that each can have a desirable net return from his activities (for example, leasing of oyster beds, and restricted licencing of nets or traps); 3. a desire to favour certain types of fishing, or certain groups of fishermen (prohibition of trawling by *large* trawlers within 12 miles of the coast, in the Maritime Provinces); 4. a desire to spread out landings over the year to promote orderly marketing of fresh fish (separate "summer" and "winter" quotas for prairie lakes); 5. a desire to restrict the duration of fishing so as to avoid stormy weather (prohibition of winter halibut fishing in the north Pacific) or so as to work only when the fish are concentrated and easy to catch; 6. a desire to obtain a more valuable product by increasing the average size of fish caught (special size restrictions on lobsters in certain areas), or by catching fish only when they are of prime quality (for example, salmon or herring before their fat is converted to sexual products).

The second type of regulation, often called *biological*, has to do with obtaining maximum sustained yield from the stock, or some similar objective. Regulations of this type are distinguished by the fact that they try to promote the economic objective of increased yield, or a larger catch per fisherman, by way of a manipulation of the size of the stock or its age composition. In their economic aspects, these so-called "biological" measures tend to benefit all fishermen indiscriminately, rather than the interests of particular groups. They are often described by names that have a certain emotional content, like "protection" or "conservation"[1] or "prevention of depletion".

[1] *I am not, of course, deprecating conservation. Using the word in its strictest sense, there is a place and a need for conservation of various fishes and of other animals and plants from the forays of man, almost everywhere on the globe.*

FRB No. 535

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The actual kinds of biological regulation vary somewhat. With salmon, which in Canada are mostly caught only toward the end of their growth, effort is devoted mainly to getting the best number of spawners on the redds—not too few and not too many—and getting them there at the proper time. With species like cod or flatfishes, where the individual fish can be available over several years of its life, the problem of maximizing yield has two aspects. For any given number of fish entering a fishery, and given amount of fishing gear, a best minimum size of fish caught can be computed from existing rates of growth and natural mortality, which will permit maximum catch from that group of fish. Existing restrictions on net size in the North Sea and parts of the northwest Atlantic are based mainly on computations of this type. However, the amount of recruitment, also, can vary with stock size or average fish size, as can rate of growth and possibly natural mortality rate; and these effects may modify or might even reverse the expected catch trend computed from existing conditions. We need to consider not only best use of available recruits, but also maintaining and increasing the production of recruits; and compromise may be necessary among conditions which favour each goal.

Before leaving these generalities, we might notice that a given regulation may sometimes serve both direct economic purposes and also the less direct economic goals which have a biological background. This is fair enough, as long as there is agreement that both ends are desirable. What is not so desirable, but quite common nonetheless, is for the biological aspects of a regulation—the "general good"—to be made a front for promoting the interests of particular groups of fishermen. I recall how the salmon traps of Puget Sound were abolished in 1935 by a public referendum or "Initiative" in the State of Washington, which had the combined support of the salmon seiners and the sport fishermen. Among the arguments put forth was the contention that the traps were a particularly destructive and pernicious type of gear, that they "didn't give the fish a chance", though actually they were taking far less salmon than the seines did. In the same vein, sea fishermen will maintain that it is shameful for salmon to be caught after they get into a river, and river anglers consider it positively sinful to take salmon from spawning beds —though the salmon are just as dead when caught in one place as in another. It would be foolish to conclude from this that all kinds of fishing are equally desirable, from the point of view of economics and public policy: however, each method should be rated on its own relative contribution to income and to satisfaction, rather than on the alleged destructiveness of competing methods.

Characteristics of Catch Quotas

So much for regulation in general. One method of regulation is to establish a catch quota, and at this meeting we are interested in quotas particularly. On the non-biological side, *daily* quotas for individual sport fishermen are a very popular device for sharing the wealth, though quite often they are ineffective because they are made so large that few fishermen ever reach them. Individual boat quotas are also sometimes applied in commercial fisheries, usually by mutual agreement rather than by law, in order to share markets or processing facilities in times of temporary glut.

However, the term quota usually refers to a limit set on the total catch taken from a stock during a season. What are the peculiar advantages and disadvantages of such quotas? Now it happens that one of the most spirited eulogies of quota regulation has been made by Professor F. I. Baranov (1947). He refers to quotas as the "American" system of fishery management, in contrast to the "European" system of closed seasons, closed areas, gear restrictions, etc. Furthermore, his knowledge of and affection for quotas is based on accounts of their operation in two Canadian fisheries the whitefish fisheries of some of our prairie lakes, and the eastern Pacific halibut fishery which is shared by Canada and the United States. I can do no better for quotas than to quote some of his remarks.

"All restrictions on fishing," says Baranov, "are useful only insofar as they lead, by one means or another, to a decrease in the catch. In that event it is simpler, more logical and more honest to limit the size of the catch directly, without stooping to petty interference with fishing techniques." Further, "once a quota is established, all other restrictions on the fishery merely increase costs for the operators, without doing the fish stocks any good." "The work of regulatory authorities is simplified [by quotas] . . . and it is possible to reduce the enforcement apparatus. The work of the fishermen is also simplified. They can use the greatest fishing power of their nets, and they can devote their means and energy not to multiplying fishing apparatus in pursuit of an impractical level of catch, but in a restriction and rationalization of this apparatus for attaining the highest economic return from the established quota."

Contrasting quotas with other methods, Baranov notes that "properly speaking, the majority of traditional restrictions on fishing are also directed toward decreasing the intensity of fishing." "But," he says, "they do not achieve that goal. Prohibition of one kind of fishing results in the fishermen turning to a different, still-legal, one, so that the amount of fishing is not reduced. Establishing a limit for size of nets . . . is followed by an increase in their number. Closure of one region to fishing is compensated by increased activity in another. Introduction of a closed season on fishing means that fishing becomes greater during the remainder of the year."

There is obviously much truth in these various points, and it would be easy to document them by illustrations from many fisheries. Yet anyone who is close to fishery administration knows that regulation by quota is not always and everywhere as simple and as beneficial as Baranov's words make it sound.

An important technical problem is the need for a highly developed statistical system which provides day-by-day information on catch taken, especially as the quota limit is approached. Such a system can be developed, and it has in fact been developed for the British Columbia herring fishery and salmon fisheries, for example; but it is expensive. If fishing success is more predictable, and if the number of boats that will fish can be estimated fairly accurately, then up-to-the-minute statistics are not essential; an estimate of the probable catch per day can be made in advance with sufficient accuracy, and the fishing season can be made of a length to conform to the desired quota.

In some fisheries neither of the above methods of approximating a quota may be available, or there may be other conditions which make an overall quota dangerous or impractical. For example, a quota covering a broad area may lead to overexploitation

of nearby grounds and underexploitation of distant ones: since the fishermen, each competing for a share of the quota, will make their cruising time as short as possible. This can be alleviated by setting individual quotas for individual subareas; but when this is done there immediately arise those problems of supervision and enforcement which quotas are supposed to obviate. Still other special conditions may make the use of overall quotas unrealistic, particularly where successive populations of a migratory fish proceed through a fishing area.

Furthermore, when you get down to cases, the difference between a quota system and other methods of regulation is by no means clear-cut. Even in Baranov's example of the Pacific halibut, we find that the actual regulations specify a certain fishing season or seasons, not a certain total catch for the year. The length of the season is of course estimated to conform to the desired catch; but, ideally at least, *any* regulation of season or time of fishing is made with a view to adjusting the catch to the most desirable level. Thus the difference between the "traditional" methods of regulation, and the so-called "American" method of regulating by quotas, becomes tenuous and indistinct.

However I do not suggest that the distinction disappears altogether. One thing which a quota brings into sharp focus is the question of the productivity of the stock. If a quota is set at 50 million pounds, for example, the first question a fisherman asks is, why is it 50? Why not 60? or 40? For that matter, how do you know that there will actually be 50 million pounds of fish on hand? Such questions are pertinent with *any* kind of regulation. But when a numerical quota has to be announced, they have a special urgency; there is no avoiding them by vague reference to a "need for conservation" or "protection of spawners". I think we can fairly claim as an advantage for quotas, over other types of regulation, that they more urgently demand information on stock size and *productive potential*, and that they stimulate efforts to determine these[2].

British Columbia herring quotas

I have been asked to indicate the status of the quotas on herring catches in British Columbia, in relation to the general question of regulation by quota. A good general summary of this matter is given by Taylor (1955). Catch quotas were introduced to a part of the British Columbia herring fishery first during the 1936-37 season. Prior to this time important fishing areas east and west of Vancouver Island had been closed to fishing for herring to be used for reduction purposes—the feeling being that there was greater economic return in using the herring for human food. However the demand for food herring had decreased, and the Province consented to wholesale reduction provided a quota was set to avoid possible excessive exploitation. The quotas set were below the known maximum catches but greater than the minimum catches for the areas concerned.

The supply of British Columbia herring varies from year to year, but usually only moderately: in this respect they are intermediate between halibut and salmon. Nowadays only two or three age-groups occur in the catches to any important degree. Success

[2]*Note especially that knowing the size of the stock is not enough. Too much preoccupation with size of stock can lead to the conscious or unconscious assumption that a large stock is an end in itself. Both theoretically and from experience we know that this is not so. It is possible for a stock to be too great to provide maximum yield, as well as too small.*

of reproduction varies considerably from year to year—more in some stocks than in others, but nowhere nearly as much as in, for example, the Norwegian herring stocks. If there are two poor year-classes in succession it means poor fishing two to three years later, while successive good reproductions set the stage for an abundant catch. In addition, in certain areas the stock is more vulnerable to capture in some years than in others. Mainly because of this last, predictions of each year's supply are not yet reliable enough to be used to set a special quota in each area for each year in advance; instead there is nominally the same quota for all years.

In practice, when herring are relatively scarce the quota is not taken. When they are abundant, nearly always a quota extension is granted. The result is that the quota sets a limit to the catch only about once in three years, and even when it does, usually only a few days remain in the season and not much more herring could be taken anyway.

However the herring stocks and fishery are far from being unregulated. There exists a closed season during spawning time, which protects the fish at a time when they are massed into very dense schools. Appropriate gear could make very large catches at such times, and could increase the total seasonal rate of exploitation of the stock from the present 45-55 per cent up to possibly 80 per cent, 90 per cent, or even higher.

In summary, the British Columbia herring fishery is producing an excellent annual yield and gives every appearance of being in a healthy state. But it is *not* a good example of the effects of regulation by quota, since the quotas to-day do not often or very greatly restrict fishing: restriction is accomplished by the closed season. However, the quotas may have potential value as a standby, in case pre-spawning fishing were ever to become substantially more effective than it is to-day.

Effects on fishermen and the industry

I do not intend to consider the economic and sociological effects of quota regulation in any detail. However it seems necessary to comment on one of the statements quoted earlier: that the use of quotas makes it possible for the fishermen to restrict and rationalize their fishing, by eliminating gear in excess of what is needed to take the quota. This of course is possible if quotas are set for individual fishermen or boats, and this system has been used in the whitefish fishery of, for example, Lake Winnipeg. A disadvantage, possibly, of this practice is that it may tend to discourage individual initiative among the fishermen (since the more skilful or enterprising ones can take no more than others) and it relaxes normal incentives to improve fishing equipment and to exploit fully the whole area from which a catch can be taken.

On the other hand, if only an over-all quota is assigned and there is no limit on total gear in use, each fisherman is exposed to the full rigours of competition—something he may not altogether care for. The general economic situation is then the same as for other common-resource operations, whose advantages and disadvantages will be evident from the remarks of the speakers who follow.

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

II. CHOLESTEROL, FAT, PROTEIN, AND WATER IN THE FLESH OF STANDARD FISH[1]

D. R. Idler and I. Bitners

Abstract

The absolute changes in free and esterified cholesterol, fat, protein, and water are discussed for the flesh of standard male and female salmon during the spawning migration. The data were obtained for 12 groups of male and 12 groups of female sockeye representing 216 fish. Fish were caught at three locations: below the entrance to the river, 250 miles up the river, and at the spawning grounds, which were 715 miles from the first sampling station.

Introduction

Recently, a pure race of Fraser River sockeye salmon was sampled at the beginning, at an intermediate point, and at the end of a 715-mile spawning migration (1). The data showed that the large number of fish taken (216) and the uniformity of the population permitted an accurate standard fish to be calculated for each sex. In effect, the realization of a standard fish permits one to obtain the data which would be obtained if a fish could be analyzed, then returned to the water and recaptured at a later time. By this technique not only percentage changes but absolute weight changes in tissue components may be determined. This report will consider the absolute weight changes in the flesh fat, protein, water, and cholesterol levels in standard male and female sockeye salmon as they migrate 250 miles from Lummi Island to Lillooet and then a further 460 miles to the spawning grounds at Forfar Creek.

Methods

The sockeye salmon were caught in reef nets and transported to the laboratory at the temperature of dry ice as previously described (1). The fish were thawed overnight and the frozen viscera including the kidneys were removed. The head and tail were severed and the well-scraped skin and bones were separated from the body flesh. The flesh of males and females was separated and divided into groups as previously described (1). The frozen flesh was put through an electric cutter and the entire sample (15 to 42 lb.) was thoroughly mixed, and representative 1-lb. samples sealed in enamelled cans and either stored at $-30°$ C. or heat processed. The heat-processed samples were employed for the analyses described in this report.

Fat was determined by a rapid method developed specifically for canned salmon (2). Water was determined with an automatic infrared moisture balance. The fat was then taken up in acetone:ethanol, 1:1 and free and esterified cholesterol determined by the Sperry–Webb procedure (3).

[1]Manuscript received April 9, 1958.
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F.R.B. No. 536

Results and Discussion

The proximate analyses for the male and female fish at each location are shown in Table I. The original moisture content and the percentage increase in moisture in the flesh was essentially the same for both sexes. The females began the spawning migration at Lummi Island with 11% more fat than the males and up to Lillooet (250 miles) both sexes appear to have lost about the same percentage of the initial fat content resulting in a 16% greater loss for the female because of the higher fat content at Lummi Island. Both sexes began the migration with the same percentage of protein and appeared to lose the same percentage in the first 250 miles. Over the entire migration distance the females used approximately 18% more protein than the males. *The percentage composition data do not show the true picture for the fat and protein consumption of the two sexes because they make no allowance for changes in the total weight of the flesh as the fish move up the river.* This point is demonstrated in Table II of the preceding paper (1) where it is shown that the body flesh of a standard male sockeye salmon decreases from 1467 g. at Lummi Island to 1300 g. at Lillooet and Forfar Creek. The flesh of the standard female sockeye weighed 1335 g. at Lummi Island, 1135 g. at Lillooet, and only 934 g. at Forfar Creek. Table II shows the actual fat, protein, and water in the flesh of an average standard male and female salmon on a weight basis at each of the three locations. Table III shows these data in a more easily interpreted form based on the changes in total weight of fat and protein per unit distance.

TABLE I

PROXIMATE ANALYSES AND CHOLESTEROL CONTENT OF THE FLESH OF MIGRATING SOCKEYE SALMON*

Components	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Moisture, %	♂	67.2±0.11(23)†	70.7±0.23(40)†	78.2±0.23(25)†
	♀	67.0±0.16(33)†	71.0±0.12(40)†	78.0±0.07(55)†
Fat, %	♂	9.34±0.24	5.07±0.24	3.23±0.23
	♀	10.55±0.12	5.59±0.19	2.70±0.11
Protein N,‡ %	♂	3.508±0.047	3.283±0.036	2.684±0.039
	♀	3.518±0.107	3.385±0.061	2.835±0.020
Moisture+fat+protein, %	♂	98.47	96.29	98.21
	♀	99.53	97.75	98.42
Free cholesterol, mg./100 g.	♂	25.7±0.81	30.9±0.22 0.01§	31.1±0.40 0.8§
	♀	25.6±0.24	30.6±0.22 0.001§	34.7±0.77 0.001§
Total cholesterol	♂	29.2±0.68	34.1±0.27 0.01	36.7±0.77 0.05
	♀	29.0±0.48	32.6±0.24 0.01	35.9±0.40 0.01

*± Standard errors.
†Total number of fish divided into four groups.
‡Total flesh protein nitrogen was determined in connection with a study on the distribution of nitrogenous components of flesh (6).
§Significance of difference from value immediately to left.

TABLE II

CHANGES IN FAT, PROTEIN, WATER, AND CHOLESTEROL IN THE FLESH OF MIGRATING SOCKEYE SALMON OF STANDARD LENGTH

Components, g. in flesh	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
Moisture	♂	986	921	1020
	♀	894	806	729
Fat	♂	137	66	42
	♀	141	64	25
Protein	♂	322	267	219
	♀	294	240	165
Free cholesterol	♂	0.377	0.402	0.406
	♀	0.342	0.347	0.324
Total cholesterol	♂	0.428	0.444	0.479
	♀	0.387	0.370	0.335

TABLE III

EXPENDITURE OF FLESH FAT AND PROTEIN BY MIGRATING SOCKEYE SALMON OF STANDARD LENGTH

Location	Sex	Component expended by standard fish in mg./mile	
		Fat	Protein
Lummi Island to Lillooet	♂	284	220
	♀	304	216
Lillooet to Forfar Creek	♂	52	104
	♀	85	163
Lummi Island to Forfar Creek	♂	132	144
	♀	161	180

The changes in fat for the standard fish are quite different than when considered on a percentage basis. The standard females use only 7% more fat than the standard males over the first 250 miles and they use an almost identical weight of protein. Since the initial weight at Lummi Island of the standard female fish (2376 g.) is 92.5% of the initial weight of the standard male fish (2565 g.) the female expends 15.3% more flesh fat and 6.5% more flesh protein than a male fish of the same body weight. The most significant difference in male and female fish over the 250 miles from Lummi Island to Lillooet is the change in the weight of gonads. The male milt increases by only 25.5 g., whereas the female roe increases by 57.9 g. (1). The female would therefore be expected to consume more body fat and protein if the energy expenditure for swimming was the same for both sexes. The data appear to substantiate this supposition. During the final 460 miles the standard females use 57% more flesh protein and 63% more flesh fat per unit distance than does the

standard male. The standard male at Lillooet weighed 2371 g. and 2583 g. at the spawning grounds, whereas the standard female weighed 2156 g. at Lillooet and 2168 g. at the spawning grounds (1). When the average weight of the standard fish for each sex is considered over this 460 miles the standard female expends 87.2% more flesh fat and 80.6% more flesh protein than does a standard male of the same body weight. The most significant difference in the two sexes over the final 460 miles of the spawning migration is in the weight of the gonads. The male milt increases by only 3 g., whereas the female roe increases by 160 g. An approximate calculation based on 17.6% protein and 9.2% fat for the ovaries (4) shows that the 157-g. excess of gonad produced by the female as compared to the male represents approximately 27.6 g. of protein and 14.5 g. of fat. Table II shows that the standard female expends 15 g. more fat and 27 g. more protein than the standard male over the last 460 miles. This excellent agreement appears to offer convincing evidence that the standard female and male sockeye salmon expend nearly identical energy for swimming and the excess flesh fat and protein consumed by the female can be attributed to the larger weight of the gonads. The standard female expends 22% more flesh fat and 25% more flesh protein than does the standard male over the 715-mile migration route (Table III). The average weight of a standard male fish from Lummi Island to the spawning grounds is 2574 g. as compared to 2272 g. for the female (1). When this weight difference is taken into account the standard female expends 38% more flesh fat and 42% more flesh protein than a standard male of the same weight. On the same equal weight basis the standard female begins the migration at Lummi Island with 10.2% more fat and 1.2% less protein. The demands on flesh fat and protein are considerably greater for the female than for the male and only in the case of fat are somewhat greater reserves available.

For the first 250 miles the standard males expend 29% more fat than protein and the females expend 40.5% more fat. For the last 460 miles the utilization reverses and the standard male expends 100% more protein than fat while the standard female expends 92% more protein (Table III).

Paton, in referring to water changes in the flesh of migrating *Salmo salar* states that "it is this increase in the percentage water of the flesh which maintains the weight of the fish per fish of standard length, although the solids as a whole have diminished" (5). This thought is prevalent in the literature in the more restricted sense that the percentage of fat plus water is a constant. There is no doubt that the percentage of water does increase as the percentage of fat decreases but the statement quoted above is not valid for the present study. The flesh of neither sex takes on water in sufficient quantity to replace depleted fat and protein reserves. Table I shows that even on a percentage basis the fat plus water at different points for the same sex are not very constant. Over the 715-mile route the flesh of the standard female lost 129 g. of protein, 116 g. of fat, and lost (not gained) 165 g. of water (Table II). The flesh of the standard males lost 103 g. of protein, 95 g. of fat, and only gained 34 g. of water, resulting in a loss in the weight of the flesh of 164 g. as compared

to 410 g. for the standard female. Table I shows that, in general, fat, protein, and water total nearly 100%. However, the data for both sexes strongly suggest that another flesh component (or components) is more significant at Lillooet than at Lummi Island or Forfar Creek. In the present study the standard eviscerated male fish which weighs 2322 g. at Lummi Island and 2382 g. at Forfar Creek does confirm Paton's statement that the weight of the fish remains essentially constant but the constancy is due only partly to the increased water in the flesh but primarily to gain in weight of the head, skin, bones, and tail from 881 g. to 1057 g. (1). The head, skin, bones, and tail of the female increases from 785 g. at Lummi Island to 834 g. at Forfar Creek, but the magnitude is not sufficient to maintain the weight of the standard eviscerated female which drops from 2151 g. at Lummi Island to 1775 g. at Forfar Creek (1).

Both the male and female standard fish showed a loss of water for the first 250 miles of the spawning migration. The loss was 6.6% for the males and 9.8% for the females. Over the last 460 miles the males gained 10.7% water while the females lost 9.7%.

Table I shows the changes in free and total cholesterol expressed in mg./100 g. of flesh. The free cholesterol per unit weight of flesh of females increased 19.5% in the first 250 miles and a further 13.5% in the last 460 miles for an over-all increase of 35.6% during the migration. However, when the loss in weight of the total flesh of female standard fish is considered (1) the total free cholesterol of the flesh increases only insignificantly (1.5%) (Table II) in the first 250 miles and decreased 6.5% over the last 460 miles for an over-all loss of 5.3% during the migration. The males showed an increase of 20% in free cholesterol per unit weight of flesh in the first 250 miles and no change after this point. The flesh of the standard male decreases from 1467 g. to 1302 g. over the first 250 miles and as a result the increase in free cholesterol per standard fish is only 6.6% . The weight of the male flesh does not change during the last 460 miles, neither does the percentage cholesterol.

The total cholesterol increased steadily for both sexes over the migration route when the results are considered per unit weight of flesh (Table I). However, when changes in flesh weight are taken into consideration the standard male fish showed a gain in total cholesterol of 12% and the standard female a loss of 13% over the 715-mile migration.

The esterified cholesterol represents only 12% of the total at Lummi Island for the females and this decreased to 6.1% at Lillooet and 3.3% at Forfar Creek. The male flesh had 12% of the total cholesterol in an esterified form at Lummi Island, 9.4% at Lillooet, and 15% at Forfar Creek.

The preceding paper showed the very considerable decreases in plasma cholesterol for both sexes over the migration route (1). It seems surprising that while flesh reserves of fat and protein and plasma cholesterol were so extensively depleted the cholesterol content of the flesh of both sexes actually increased per unit weight of tissue. In the case of the male even the standard fish showed an increase in flesh cholesterol while the standard female suffered

no great loss. It should be emphasized, however, that whereas the flesh of a standard male fish contained 322 g. of protein and 137 g. of fat at Lummi Island, it only contained 428 mg. of cholesterol (Table II). Thus, from an energy consideration, it makes little difference whether the fish does or does not utilize the flesh cholesterol.

Acknowledgment

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

III. CHANGES IN THE PROTEIN AND NON-PROTEIN NITROGEN FRACTIONS IN MUSCLES OF MIGRATING SOCKEYE SALMON[1]

D. W. Duncan and H. L. A. Tarr

Abstract

The nitrogen content of three protein fractions and one non-protein nitrogen fraction of muscles of migrating sockeye salmon was determined at three points on the migratory route. The results have been expressed as milligrams of nitrogen per 100 g. of muscle, as per cent of the total muscle nitrogen, and as grams of nitrogen per average (standard) male or female fish.

Introduction

The only previous studies in which determinations of changes in total protein, water-soluble nitrogenous constituents, and lipids in muscles of migrating salmon were made are those of C. W. Greene (1) and C. H. Greene (2) with "spring salmon" (*Oncorhynchus tschawytscha*). They studied only a single protein fraction which was prepared by heat coagulation and solvent extraction of the whole muscles. It was found that the protein content of the wet muscle tissue decreased from 20.07 to 14.02%, and that the water content increased from 74.8 to 81.5% as the fish migrated from the river mouth to the spawning grounds (1). The total water-soluble extractive nitrogen increased slightly, the amino nitrogen content increased markedly, and the creatine nitrogen remained practically unchanged during migration (2). In the present investigation changes in three protein fractions and in the non-protein nitrogen of the muscles of migrating sockeye salmon (*Oncorhynchus nerka*) have been determined.

Materials and Methods

The methods of securing representative samples of fish at three points on the migratory route and of preparing the muscle fraction from these have been described in detail (3).

The protein fractionation procedure was based on that which has, with various modifications, been used to prepare protein fractions from mammalian or fish skeletal muscles for many years (4). It involved preparation of four general fractions, namely (1) proteins soluble in cold, approximately neutral salt solution of comparatively high ionic strength and insoluble in salt solutions of low ionic strength (largely actomyosin); (2) proteins soluble in cold neutral salt solutions of low ionic strength ("myogens", "myoalbumens", etc.); (3) proteins insoluble in the above solutions (collagen and elastin; denatured myosin); and (4) nitrogenous compounds not precipitated by cold 5% trichloroacetic acid solution (non-protein nitrogen).

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F.R.B. No. 537

TABLE I

NITROGEN PARTITION IN PROTEIN AND NON-PROTEIN NITROGEN FRACTIONS OF MUSCLE TISSUE OF MIGRATING SOCKEYE SALMON

Station	No. and sex of fish per group		Mg. of N per g. (wet weight) and % of total N					
			Whole muscle	Stroma	High ionic strength	Low ionic strength	Non-protein	% recovery‡
Lummi Island	7 ♂	mg.	34.64	4.96	15.70	8.77	4.55	
		%	100	14.32	45.32	25.32	13.13	
	6 ♂	mg.	35.28	6.30	15.09	7.94	4.75	
		%	100	17.86	42.77	22.51	13.46	
	5 ♂	mg.	34.20	6.02	15.60	7.45	3.87	
		%	100	17.60	45.61	21.78	11.31	
	5 ♂	mg.	36.20	7.56	17.32	7.68	4.86	
		%	100	22.05	47.84	21.21	13.42	
	Av.	mg.	35.08±0.47	6.21±0.53	15.93±0.58	7.96±0.287	4.43±0.223	
		%	100	17.96±1.66	45.39±1.04	22.69±0.825	12.83±0.51	98.43
	8 ♀	mg.	32.00	8.40	12.40	7.47	4.83	
		%	100	26.25	38.75	23.34	15.09	
	7 ♀	mg.	36.60	7.00	15.81	7.81	4.07	
		%	100	18.13	40.96	18.94	10.54	
	9 ♀	mg.	36.20	7.72	14.90	8.16	4.40	
		%	100	21.33	41.16	22.54	12.15	
	9 ♀	mg.	35.90	5.68	16.68	8.82	4.64	
		%	100	15.82	46.46	24.57	12.92	
	Av.	mg.	35.18±1.07	7.20±0.68	14.95±0.15	7.94±0.288	4.48±0.165	
		%	100	20.55±2.26	42.60±1.64	22.60±1.21	12.61±0.945	98.36
	10 ♂	mg.	33.71	4.50	15.39	9.30	4.58	
		%	100	13.35	45.65	27.59	13.59	
	10 ♂	mg.	32.27	4.42	14.47	8.82	4.70	
		%	100	13.70	44.84	27.33	14.56	
	10 ♂	mg.	33.15	5.36	14.58	9.94	4.71	
		%	100	16.17	43.98	29.28	14.21	
	10 ♂	mg.	32.20	5.25	14.46	9.13	4.74	
		%	100	16.30	44.70	28.35	14.72	
	Av.	mg.	32.83±0.36*	4.88±0.44	14.73±0.20	9.30±0.226†	4.68±0.041	
		%	100	14.86±0.785	44.80±0.345	28.33±0.44†	14.25±0.25†	102.24

Lillooet	10	♀	mg.	34.72	5.44	15.30	9.63	4.60	
			%	100	15.67	44.07	27.73	13.25	
	10	♀	mg.	34.71	4.95	14.11	9.16	4.91	
			%	100	14.26	40.65	26.39	14.15	
	10	♀	mg.	32.12	4.97	14.42	9.16	4.56	
			%	100	15.47	44.90	28.52	14.20	
	10	♀	mg.	33.87	5.27	14.23	9.25	4.37	
			%	100	15.56	42.01	27.31	12.90	
	Av.		mg.	33.85±0.61†	5.16±0.48†	14.52±0.92	9.30±0.111†	4.61±0.111	
			%	100	15.24±0.328	42.91±0.965	27.47±0.44†	13.62±0.323	99.24
	10	♂	mg.	27.65	4.18	13.05	6.41	4.25	
			%	100	15.12	47.20	23.18	15.37	
	5	♂	mg.	26.83	4.07	13.30	6.41	4.26	
			%	100	15.11	49.57	23.89	15.88	
	6	♂	mg.	27.11	4.14	13.39	6.49	4.02	
			%	100	15.27	49.39	23.94	14.83	
	4	♂	mg.	25.78		13.22	6.33	3.88	
			%	100		51.28	24.55	15.05	
	Av.		mg.	26.84±0.39*	4.13±0.23	13.24±0.27*	6.41±0.240*	4.10±0.091	
			%	100	15.30	49.33±0.84	23.88±0.28*	15.28±0.23†	103.69
Forfar	14	♀	mg.	27.86	3.46	13.49	6.33	4.83	
			%	100	12.42	48.42	22.72	17.34	
	14	♀	mg.	28.20	4.32	13.81	6.73	4.51	
			%	100	15.32	48.97	23.86	15.99	
	13	♀	mg.	28.58	4.31	12.86	6.99	4.80	
			%	100	15.08	45.00	24.42	16.79	
	14	♀	mg.	28.78	4.06	12.27	6.88	4.37	
			%	100	14.11	42.63	23.90	15.18	
	Av.		mg.	28.35±0.20*	4.04±0.086†	13.11±0.34	6.74±0.250*	4.63±0.224	
			%	100	14.25±0.655	46.24±1.50	23.75±0.357*	16.33±0.47†	100.57

*Significance of difference from the corresponding preceding average value = $>t$ 0.01.
†Significance of difference from the corresponding preceding average value = $>t$ 0.05.
‡Average nitrogen recovered in the four fractions as per cent of total nitrogen.
NOTE: Means are tabulated ± standard error.

Salmon muscle (20 g.) was blended in a tightly closed, completely filled, ice-packed stainless steel jar of a Servall Omni-Mixer with 380 ml. of buffered 0.85 *M* NaCl solution (5). An aliquot (250 ml.) of the suspension was centrifuged 30 minutes at 20,000 *g* and 0° C. The supernatant was decanted and the residue (stroma proteins and denatured myosin) dissolved in 25 ml. of 1 *N* NaOH. After storage overnight at 10° C., the solution was adjusted to 50 ml. and 5-ml. aliquots were used for nitrogen determinations. The supernatant liquid was adjusted to 250 ml., and a 100-ml. aliquot was mixed with 900 ml. of water at 0° C. and, after 16–18 hours at 0° C., 350 ml. of the suspension was centrifuged at 20,000 *g* and 0° C. The centrifugate was dissolved in 25 ml. of 1 *N* NaOH and adjusted to 50 ml. with water for nitrogen determinations, using 10-ml. aliquots (high ionic strength fraction). The supernatant liquid was readjusted to 350 ml. with water, and to a 100-ml. aliquot 5 g. of trichloroacetic acid was added with stirring at 0° C. The solution was centrifuged 30 minutes at 20,000 *g* and 0° C. The volume of the supernatant solution was determined, and duplicate 25-ml. aliquots were used for nitrogen determinations (non-protein nitrogen fraction). The trichloroacetic acid precipitate was dissolved in 12 ml. of 1 *N* NaOH, the solution adjusted to 25 ml., and its nitrogen content determined using 10-ml. aliquots (low ionic strength fraction).

Nitrogen was determined by the modification of the Kjeldahl procedure described by Campbell and Hanna (6).

Results

The conditions under which the fish were obtained and the samples were prepared, which involved slow freezing, and thawing, and mincing the flesh in a "silent cutter" (3) undoubtedly caused considerable denaturation of the myosin. The large number of fish sampled, and the transportation distances involved, made it virtually impossible to carry out the fractionation procedure under conditions which would preclude myosin denaturation. In the procedure used denatured myosin would be found in the stroma fraction, and that this is the case is borne out by the finding that the stroma protein fraction accounted for from 14.25 to 20.55% of the total muscle nitrogen, values greatly in excess of the values (3 to 4%) which are considered normal for teleost fish (7). If it is assumed that true stroma proteins account for 3% of the total muscle nitrogen, and that the remaining nitrogen is denatured myosin, then the "high ionic strength" fraction would account for 54.15 to 61.6% of the total nitrogen instead of the 42.6 to 49.33% actually found. The higher values are similar to those reported for fish actomyosin in the literature (4, 8).

When calculated on the basis of milligrams of nitrogen per gram of muscle (per cent basis) (Table I) there is a significant decrease in the total nitrogen content of the fish as they migrate. This decrease is largely due to decreases in the nitrogen content of the high ionic strength plus stroma protein fractions. The concentration of nitrogen in the low ionic strength fraction increased significantly between Lummi Island and Lillooet, and decreased significantly

TABLE II

NITROGEN CONTENT OF MUSCLES OF MIGRATING SOCKEYE SALMON (GRAMS PER STANDARD FISH)

Fraction	Males			Females		
	Lummi Island	Lillooet	Forfar	Lummi Island	Lillooet	Forfar
Whole muscle	51.462	42.754	34.951	46.966	38.376	26.717
Stroma + high ionic strength	32.479	25.540	22.038	29.470	22.329	16.149
Low ionic strength	11.677	12.111	8.354	10.769	10.554	6.347
Non-protein nitrogen	6.499	6.097	5.340	5.988	5.225	4.362

between Lillooet and Forfar. The significant increase in the percentage of nitrogen in the non-protein fraction is due to the significant decrease in the total nitrogen of the samples. The actual composition of the fraction itself in terms of milligrams of nitrogen remained constant within limits of error, and this is verified by the detailed analysis of this fraction (9).

The amount of nitrogen in each of the four fractions when calculated on the basis of the weight of a "standard" fish (3) is recorded in Table II. The values given in this table were obtained by multiplying the average nitrogen concentrations shown in Table I by the weights of standard male or female fish. The marked decrease in the nitrogen of the whole muscle is due to both the marked decrease in weight of the fish and to the lower per cent nitrogen content of the muscle as migration proceeds. The sum of the nitrogen of the high ionic strength and stroma protein fractions also decreases but the decrease is much more pronounced for females than males during the later stages of migration. Initially there is practically no change in the amount of nitrogen available to the fish in the low ionic strength fraction but a sharp decrease occurs during the later stage of migration. The decrease in non-protein nitrogen is entirely due to decrease in weight of the fish and not to the nitrogen concentration of the fraction.

Acknowledgment

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THE MANNER IN WHICH THE SPONGE CLIONA BORES IN CALCAREOUS OBJECTS[1]

FREDERICK E. WARBURTON[2]

Abstract

Boring in shells and calcite crystals was investigated, using a new type of reconstitution culture. No free acid was detected, and no detectable quantity of calcium was put in solution by the sponge. However, acid action is indicated by restriction of boring to calcium carbonate. Conchiolin is penetrated with difficulty. Cells in contact with the substratum form a reticulum of fine pseudopodia and filaments; a corresponding pattern of lines is etched into the calcite. The areas so marked out are the same size and shape as the fragments discharged by the sponge. These fragments are identical whether from shells or calcite crystals; their faces are curved but their edges sharp. Apparently the cytoplasmic filaments insinuate themselves into calcite by secreting minute amounts of acid, and undercut fragments which are carried through the sponge parenchyma to excurrent canals.

Cliona is a cosmopolitan genus of marine siliceous sponges (F. Clionidae, O. Hadromerina, Cl. Demospongiae), remarkable for the habit of living in tunnels and galleries bored in limestone, coral, and the shells of molluscs. The damage inflicted by *Cliona* on various mollusc fisheries and on limestone breakwaters invests the genus with some economic importance, and sponge-riddled shells and stones are familiar objects to the naturalist on the seashore. The manner in which the galleries are excavated has therefore attracted the researches of several biologists, and the speculations of many more.

This paper will recount some of the results of an investigation into the biology of boring sponges, sponsored by the Fisheries Research Board of Canada because of the deleterious effects of the sponges on the oyster and scallop fisheries of eastern Canada. The work was carried out at the Board's Biological Sub-Station, situated on Malpeque Bay on the north shore of Prince Edward Island, near Ellerslie. Studies were largely confined to *Cliona celata*, the prevalent species in that area.

Historical Note

Grant (3), who first described *Cliona celata*, believed that his new "zoophyte" dwelt in burrows made by worms. Bowerbank (1) shared this opinion. Hancock (4,5) refuted Bowerbank's arguments, and made it clear that *Cliona* bored its own holes; he suggested that this was done by grinding with the spicules. However, Nassanov (9,10) showed that *Cliona* larvae began to bore before they possessed spicules.

Nassanov had described the fragments of shell discharged by boring sponges. These, and fragments of conchiolin from bored oyster shells, were figured by Topsent (12), who pointed out that since conchiolin is bored in the

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Contribution from the Fisheries Research Board of Canada, Biological Station, St. Andrews, New Brunswick.
[2]c/o Department of Anatomy, McGill University, Montreal, Que.

same way as shell it is difficult to accept the hypothesis that *Cliona* bores by secreting an acid. Hancock had felt that a simple organism like *Cliona* could not be expected to perform complex chemical feats like the secretion of acids. Letellier (6) found that the tissues of *Cliona* are not acid, and pointed out that the detached fragments of shell are not corroded at the edges. He proposed that contractions of the sponge tissues might set up torsions which would break off bits of the shell to which they stuck; such torsions experimentally set up with rubber rods broke off lunular fragments resembling, but larger than, those produced by *Cliona*. His hypothesis failed to receive general acceptance.

Vosmaer (13) reviewed the early work on the problem, and suggested seeking an explanation by comparing the action of *Cliona* with that of various boring algae and fungi. He also suggested that the sponge secretes an acid which dissolves calcium carbonate, and, in addition, an enzyme which attacks conchiolin. Otherwise, he was unable to do more than confirm the earlier work of Nassanov and Topsent.

Galtsoff and Pertzoff (2) titrated suspensions of the cells of *Cliona* and of *Microciona*, and found that the *Cliona* suspension bound less acid and more base than the other. Old (11) developed the extremely useful technique of cultivating *Cliona* larvae on crystals of calcite.

Since the time of Nassanov and Topsent, however, little has been published on the boring mechanism of the Clionidae except speculation—often dogmatic speculation. It has been suggested that the boring is done by an alga or fungus symbiotic with the sponge; but this seems to be simply a retreat from the problem. No constant association of sponge and fungus has been demonstrated; and the few boring fungi which the writer has observed in his cultures have formed rectangular reticula, which could not remove fragments of calcite shaped like those discharged by the sponge. Another suggestion was that *Cliona* does not secrete acid, but attacks shells solely by enzymatic action on the conchiolin which is supposed to bind them together; but the sponge bores perfectly efficiently in limestone and pure calcite. It has even been stated that "*Cliona sulphurea*" (a synonym of *C. celata*) bores by secreting sulphuric acid; a hypothesis with no support but linguistic association.

Materials and Methods

Spongy shells of oysters, *Crassostrea virginica*, and of other molluscs, were obtained by dredging infested oyster beds. These sponges could be kept alive indefinitely in an aquarium with constantly flowing sea water, as stock material for the study of boring.

I adopted Old's idea of culturing sponges on transparent calcite crystals. However, instead of waiting for the irregular and uncertain supply of sponge larvae, I used "reconstitution cultures". When spongy shells are broken up with bone-cutting forceps, they tend to split horizontally into large flakes, exposing masses of sponge which can be scraped off with a blunt scalpel or forceps. Fragments up to about 0.05 ml. in volume could be obtained in

this way. Such pieces were dropped into a finger bowl of filtered sea water for a few minutes, to remove loose cells which would die and foul the cultures, and then transferred by pipette to a calcite crystal, cover glass, or other substrate, in a second finger bowl. If large sponge fragments could not be procured, several small ones were piled together. The cultures had then to be left undisturbed for three or four days, without a change of water. After that time, the fragments had attached themselves to each other and to their substrate well enough to permit them to be transferred daily to new water in clean dishes.

Within two or three weeks these masses of sponge tissue had flattened themselves into discs about a millimeter thick at the center, tapering to a single cell at the edges. The largest such discs obtained were about 15 mm. in diameter, and quickly developed a complicated system of excurrent canals with oscula.

If paraffin sections were required, reconstitution cultures were removed from calcite crystals by dropping crystal and sponge into Bouin's fixative. Within 24 hours the sponge floated off the crystal, and could be embedded in paraffin and sectioned in the usual way. Calcite fragments in the specimen were destroyed by the fixative and spicules did not make sectioning difficult, although frequent knife-sharpening was necessary. After considerable experimentation, I chose Heidenhain's iron haematoxylin followed by eosin as the most generally useful stain.

For microscopic study of the early stages of the boring process I needed to remove sponges from their calcite without damaging the crystal surface; and to examine the cells in contact with the substratum, I had to remove sponges from glass cover slips on which they were growing in such a way as to leave a single layer of cells behind. I tried a variety of elegant methods, until I found that I could obtain good results by gently wiping off the sponge with my thumb.

To study the gross pattern of sponge borings in shells, wax casts of the tunnels were prepared. Previous workers have depicted borings that are exposed by breaking away surface layers of shell (Hancock, copied by Vosmaer (14); and Medcof (8)), by grinding sections of bored objects (Vosmaer (14)), by photographing thin translucent shells (Medcof (8)), or by using X-ray photographs (L. M. Dickie and P. Ghent, unpublished). None of these methods give a clear three-dimensional view of the sponge cavities.

Shells were prepared for tunnel castings by boiling in sodium hydroxide solution until free of sponge tissue, rinsing in boiling water, and draining until the cavities were almost, but not quite, empty of water. They were then infiltrated with paraffin kept melted in a boiling water bath. They were alternately subjected to sufficiently reduced pressure to boil the remaining water out of the tunnels and to atmospheric pressure to force the melted paraffin into the cavities evacuated by the steam. When the paraffin had hardened, it was scraped from the outside of the shells with a knife and wire brush, and the shell was dissolved in hydrochloric acid.

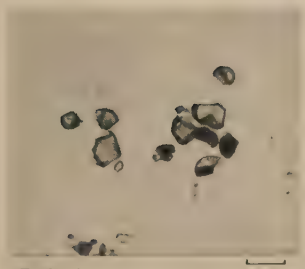
Certain experiments involved analyzing sea water in which sponges had been kept, for its content of dissolved calcium. The sea water from each experimental container was filtered and divided into two or more parts, each of which was analyzed by the usual method of precipitation with ammonium oxalate solution, re-solution in sulphuric acid, and titration with standard potassium permanganate solution.

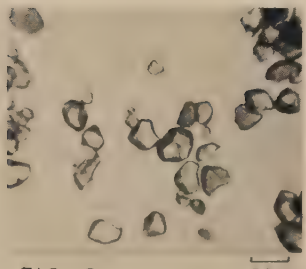
Results

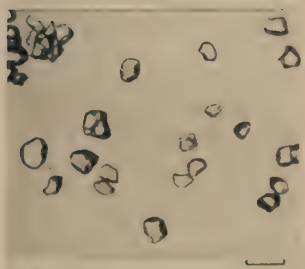
Reconstitution cultures could be grown on any clean surface which did not release toxic solutes into the water. They readily excavated cavities in several species of mollusc shells, limestone, and calcite crystals, but completely failed to penetrate the surface of any of the following substances: sandstone, brick, china, concrete, glass, bakelite, black rubber, hard rubber, celloidin, wood, paraffin wax, crab shell, and conchiolin. (The last substance was obtained in sheets inside the shells of oysters kept alive several weeks after their hinges had been dislocated.)

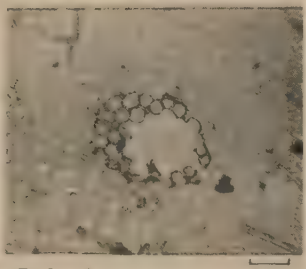
Cultures of *Cliona* on calcite were immersed in a variety of indicator solutions in sea water and examined under the high power of the microscope for signs of localized regions of low pH. No such signs were observed. Other cultures on cover glasses were inverted over depression slides filled with indicator solutions, and the thin edge of the culture observed under oil immersion; again, no color changes indicative of acid were seen. One weakness of these tests is that all indicators used were toxic in the high concentrations needed to make color changes visible in microscopic volumes. Their toxicity may have arrested those very processes they were expected to reveal. That they were toxic is shown by the fact that sponges exposed to them for an hour or two died a few days after. A critic of this report has pointed out another weakness which would have obtained even if the indicators had not been toxic. In the presence of carbon dioxide, calcium carbonate tends to become soluble bicarbonate. The reaction is reversible and the equilibrium delicate. This means that, even in the general alkaline medium of sea water, sponges may be able to produce sufficient carbon dioxide at localized sites to convert carbonate into soluble bicarbonate and erode shell or calcite. It also means that pH's at sites of erosion, in surrounding media, and in sponge tissues may all be on the alkaline side of neutrality and that differences between these pH's may be very slight indeed. If they are detectable at all, one should expect them to be revealed only by refined techniques, not by the crude methods of microscopic inspection I have depended on.

Many spicules are included in the sponge fragments used for reconstitution cultures, but these become arranged around the edge of the discs, with their points radiating outward and not in contact with the substrate. Boring may begin anywhere within the disc, and is not confined to regions containing spicules. Transverse sections of actively boring sponge cultures confirmed that no spicule points were directed toward the calcite. Also, I was able to rear a number of sponge larvae through metamorphosis on calcite crystals.









FIGS. 1–3. Views with transmitted light of unmounted fragments disgorged by *Cliona celata* boring in, (1) oyster shell; (2) quahaug shell; (3) calcite crystal.

FIG. 4. Sheet of conchiolin obtained by dissolving a spongy oyster shell in hydrochloric acid, showing a perforation, with incompletely detached marginal fragments, made by *Cliona celata*. Unstained; mounted in balsam; photographed by transmitted light.

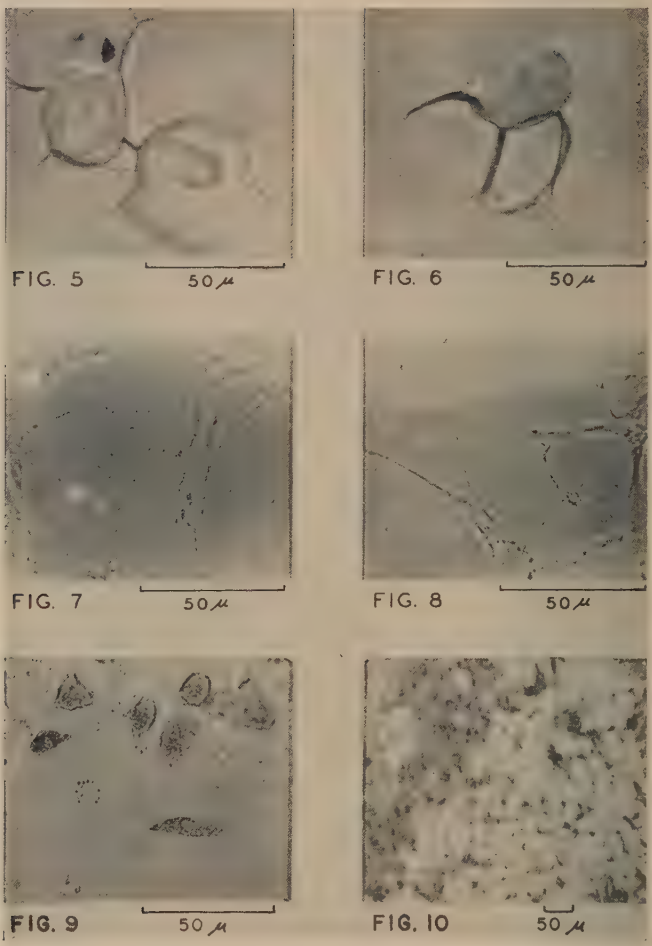


FIG. 5. Surface of a calcite crystal into which *Cliona celata* had begun boring. Three cavities left by removal of calcite fragments can be seen, and an adjacent pattern of lines etched into the crystal. Transmitted light.

FIG. 6. Another surface view of crystal shown in Fig. 5. One fragment has been removed, and outlines of more have been etched into the calcite.

FIG. 7. Living sponge cells left on a cover glass after removal of culture of *Cliona celata*, photographed by phase contrast. Note the cytoplasmic films and filaments forming figures resembling those etched into calcite (Figs. 5, 6). A few bacteria are scattered over the exposed glass. The three light spots are artifacts.

FIG. 8. Another view of living sponge cells on a cover glass from which a culture of *Cliona celata* has been removed. Cytoplasmic films and fine pseudopods are visible.

FIG. 9. Edge of living culture of *Cliona celata* on cover glass. Neutral red stain; transmitted light. The nucleus of a cell of the type which produces films and threads of cytoplasm is surrounded by a circle of stained granules, but its cytoplasm is scarcely visible. The numerous very granular amoebocytes do not appear to be directly involved in boring.

FIG. 10. Paraffin section of culture of *Cliona celata* boring in calcite, cut parallel to crystal face. Stained with iron haematoxylin and eosin. The numerous cavities contained calcite fragments which have been destroyed by fixation.

These began to bore as soon as metamorphosis was complete, before any spicules had formed, thus confirming Nassanov's (10) statement.

Fragments removed by *Cliona* from the shells of oysters (Fig. 1), from *Venus mercenaria* (Fig. 2), and from crystals of calcite (Fig. 3) are identical in shape and size. Each is bounded by several curved faces, some convex and some concave, which meet in sharp edges. Fragments of this shape are not produced when a shell is broken by pressure or hammering; and if a large crystal of calcite is reduced to powder by mechanical means, it usually breaks cleanly along planes parallel with the crystal faces, so that the smallest fragments are still typical crystals, bounded by plane surfaces and straight edges.

Most calcite crystals are marred by scratches and flaws; *Cliona* showed no inclination to attack these weak points, but began its borings anywhere on the surface of the crystal. Scratches made on shells and crystals with a needle had no influence on the location of borings.

To discover whether boring sponges dissolve calcium carbonate, I filled each of eight finger bowls with slightly more than 100 ml. of filtered sea water. In each of the four "test" dishes I placed a clean cover glass and a calcite crystal bearing an actively boring sponge; in the four control dishes I placed a clean calcite crystal, and a cover glass bearing a sponge. The dishes were covered with watch glasses, and set aside for 3 days. At the end of that time, I filtered the water from each dish and analyzed it for its calcium content.

The results of these analyses varied from 332 to 346 mg. calcium/liter; test dishes gave results both higher and lower than control dishes, but the differences between tests and controls were not generally larger than those between duplicate analyses on the same dish. During the period of this experiment, two or three of the more active sponges discharged 10 to 15 mg. of calcite fragments. Had they dissolved this amount of calcite, the calcium content of the water would have risen by more than 50 mg./liter.

When oyster shells are dissolved in hydrochloric acid, sheets of an organic substance, conchiolin, which often separate pearly layers of the shell, are left behind. These give a positive reaction with certain tests for proteins (Medcof (7)). When spongy shells were dissolved in acid the sheets of conchiolin were found to be pierced by holes bounded by partially detached flakes (Fig. 4). These were similar in shape to cross sections of shell fragments detached by the sponge (Figs. 1 and 2). Wax casts of excavations in oyster shells showed a definite layered construction, most of the tunnels lying parallel to the faces of the shells, with only a few perpendicular tunnels passing through the conchiolin sheets—just as a coal mine might have many layers of horizontal galleries in the coal, with only a few vertical shafts passing through the intervening strata of slate. Shells of living mussels were never found to be spongy until the horny periostracum had been abraded near the umbo; sponges planted on the inner surfaces of empty mussel shells attacked them readily, but those planted on the outer surface failed to pierce the

periostracum. All these observations suggest that *Cliona* does not pierce conchiolin readily. Nevertheless, in nature, boring sponges often cause severe damage to the hinge ligaments of oysters, which are of conchiolin.

The surface of a calcite crystal into which a sponge had begun to bore showed a pattern of fine curved lines, outlining areas similar in shape to those surrounding holes through conchiolin. Similar lines, not yet forming closed figures, branched from these (Figs. 5 and 6). The areas outlined by such lines were of the same shapes and dimensions as the calcite particles which the sponge disgorged. The lines were etched into the surface of the calcite, and could not be removed by polishing with fine abrasives ("Bon Ami"). No such lines were found on cover glasses on which sponges had been reared to the same age, nor were they ever found on sponge-free calcite crystals, even though these were kept in dishes with sponge cultures and became covered with the same microorganisms that surrounded the sponges.

With the phase contrast microscope, the cells left on a cover glass after a sponge had been gently wiped off showed a remarkable network of thread-like interconnections and pseudopodia, often 50 μ or more long. These surrounded areas reminiscent in shape of the calcite particles excavated by the sponges, or of the lines etched on calcite crystals. Other cells showed broad filmy expansions, only two or three microns thick (Figs. 7 and 8). These structures were hard to see with the ordinary microscope, and difficult to fix and stain. Similar pseudopodial extensions existed at the edges of reconstitution cultures, where they could be seen by ordinary microscopy; but the cytoplasm of the responsible cells was clear and transparent, and attention was distracted from it by another type of amoebocyte with a granular cytoplasm. Neutral red staining increased the difficulty of observation, as a mere ring of granules around the nucleus stained in the pseudopodial cells, while the other amoebocytes were filled with deeply staining particles (Fig. 9).

When a sponge culture on calcite was illuminated from below on the stage of the dissecting microscope, particles of calcite could clearly be seen moving with the exhalent streams through the canals at a speed of five or six centimeters per second to the oscula, where they were discharged. These particles rapidly accumulated in gleaming-white piles near the oscula, and sometimes completely surrounded them like a cone of volcanic debris. Within the parenchyma of the sponge could be seen masses of fragments, which moved perceptibly only in the course of several hours. Each of these particles was surrounded by cells, and a section through the region occupied by such a mass before acid fixation presents the appearance of foam, each cavity the size and shape of a calcite fragment (Fig. 10). The masses moved slowly toward nearby excurrent canals, into which the particles were discharged and carried away.

Discussion

In this investigation, I cannot claim to have advanced much farther toward a solution of the problem than did Nassanov and Topsent. However, my

technique of producing small sponges by the reconstitution of fragments has been exceedingly useful to me, and should be useful to others who wish to pursue the problem further.

These small sponges on calcite crystals permit the observation of many activities which cannot easily be studied otherwise, and which have not been described before; for example, movement of calcite particles through the sponge tissues to the excurrent canals. This must involve complex movements of the cells surrounding the particles. However, the movement of massive particles by sponge tissues is not rare. In reconstitution cultures of *Cliona*, spicules up to 350 μ long are rearranged and distributed peripherally with their points radiating outward. A *Cliona* culture on glass becomes surrounded by a growth of diatoms and other organisms, but a zone one millimeter or more wide remains clear because of the constant back-and-forth motion of the edge of the sponge. According to van Weel (15), particles more than 80 μ in diameter may be phagocytized by the surface epithelia of sponges.

What I have contributed to an understanding of boring justifies Nassanov's and Topsent's rejection of the idea that *Cliona* bores with its spicules, or dissolves its way through shells and stones by the copious secretion of acids.

Chemical action of some sort is strongly indicated, however, by the inability of the sponge to bore through anything but calcium carbonate, with the puzzling exception of conchiolin; and my results suggest that *Cliona* does not penetrate conchiolin as readily as Topsent and others believed. Other substances of a shell- or limestone-like consistency are unaffected by it. The obvious inference is that an acid is involved; but this must be produced in minute quantities; otherwise it would have been detected by my indicator solutions, or would have produced a noticeable increase in the calcium content of the water in which my finger-bowl cultures were kept.

Old (11) stated that "analyses of sea water containing active boring sponges in otherwise clean shells . . . show a slight daily increase in dissolved calcium". He did not describe his experimental technique, and it seems possible that the increase in calcium which he observed may be due to the action of other organisms on the shells (it is almost impossible to obtain a spongy oyster shell which is "otherwise clean") or even to evaporation of water in his containers. I feel confident that in my experiments I would have detected a consistent increase in calcium of the order of 5 mg./liter. In other words, if the sponge dissolves any of the calcium it removes, it must be less than 10% of it.

On the other hand, my results indicate that a purely mechanical explanation of boring, such as that proposed by Letellier (6), must be excluded. Calcite crystals and shells do not produce similar fragments when broken mechanically, and calcite, at least, breaks into fragments very different from those removed by the sponge.

The striking resemblance in pattern between the lines etched into calcite and the cytoplasmic reticulum underlying the sponge suggests that an acid, or some other solvent, is secreted in minute quantities by the threads of

cytoplasm. This would seem to be the only way that pseudopodia and films of cytoplasm could insinuate themselves into the calcite to excavate particles of such consistent sizes and shapes regardless of differences in substratum. I might suggest an analogy with tunnelling through limestone, not by spraying acid on it from a hose, but by carving out blocks with acid dispensed from a pipette.

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BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

IV. THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE MUSCLE[1]

J. D. Wood

Abstract

The non-protein nitrogenous constituents of muscle of migrating sockeye salmon were investigated. These constituents were found to be the same in both male and female fish and were present in approximately the same amounts in both sexes. The histidine content of the muscle in all fish decreased to one fifth of the original value during the early stages of the migratory journey and remained at the low level thereafter. Some of the other constituents changed to a smaller extent, usually increasing in the later stages of the migration. This was especially noticeable in female fish. However, the increase in the concentration of these constituents in the muscle was due to a decrease in the amount of muscle in the fish rather than to an increase in the amounts of the compounds themselves.

Introduction

Little work has been carried out on the non-protein nitrogenous constituents of migrating salmon. Greene (1) studied the muscle nitrogen extractives of king salmon, *Oncorhynchus tschawytscha*, and he found that the amino nitrogen increased during the migration and that the creatine nitrogen showed no trend either up or down. Duncan and Tarr (2) found that the non-protein nitrogen of salmon muscle did not change significantly during the migration of the fish. The individual nitrogenous constituents were investigated and the results of these studies are presented in this paper.

Materials and Methods

Muscle Samples

These were taken from the fish described by Idler and Tsuyuki (3), and were the same samples as used by Duncan and Tarr (2) for total non-protein nitrogen estimations.

Preparation of Extracts

Approximately 10 g. samples of muscle were homogenized in 3.2 volumes (v/w) absolute ethanol for 10 minutes. The resulting homogenate was kept at room temperature for 20 minutes, centrifuged at 4000 r.p.m. for 5 minutes, and the supernatant fluid decanted. The residue was resuspended for 20 minutes at room temperature in 80% ethanol equal in volume to the supernatant fluid removed. The mixture was centrifuged and the supernatant fluid collected as before. Five such successive extractions were carried out, and the supernatant fractions from these were combined and made up to 200 ml. with 80% ethanol. Aqueous extracts were prepared from the alcohol

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extracts by shaking with 3 volumes of chloroform and removing the upper aqueous layer as described by Awapara (4). Alcohol extracts were employed for the investigations unless otherwise stated.

Analyses

The α-amino nitrogen was estimated using aqueous extracts by the method of Pope and Stevens (5). Volatile-base nitrogen, trimethylamine nitrogen, and the trimethylamine oxide nitrogen were determined as described by Ronold and Jakobsen (6). Histidine was estimated by the method of Albanese *et al.* (7) with one variation; the solutions were made up to the final volume with 50% ethanol to stabilize the color. Creatinine was determined by the method of Bonsnes and Taussky (8) and creatine by that of Eggleton, Elsden, and Gough (9) as described by Ennor and Rosenberg (10).

Anserine was estimated as follows using aqueous extracts from approximately 10 g. of muscle. The diamino acids were separated from the other amino acids by means of a silicic acid column (11). The fraction containing the diamino acids also contained the anserine, which was recovered in over 90% yield. The fraction was taken down to dryness *in vacuo* and hydrolyzed in 6 *N* hydrochloric acid for 6 hours at 110° C. in a sealed tube. The solution was taken down to dryness repeatedly to remove hydrochloric acid and the residue dissolved in a small known volume of water. Samples from this, together with β-alanine standards, were chromatographed using butanol – acetic acid – water (5:1:4 v/v). The paper was dipped in a solution of 0.25% ninhydrin in acetone and the spots were developed at room temperature for 24 hours. The color density of the spots was measured in a Beckman spectrophotometer (12) and the amount of β-alanine in the samples calculated by the method of maximum density as described by Block (13). This method was applicable to the salmon muscle extracts because chromatography showed that no carnosine was present. The latter compound would otherwise have interfered since it also gives rise to β-alanine on hydrolysis. The amount of anserine in the sample was calculated from the amount of β-alanine produced by hydrolysis.

Chromatography

Two-dimensional paper chromatograms were run on appropriate amounts of muscle extract using Whatman No. 1 paper. The first solvent system was phenol–water (4:1 w/v) and the second system was collidine – 2,4-lutidine – water (1:1:1 v/w). The length of run was 25 cm. for both systems. The papers were sprayed with 0.25% ninhydrin in water-saturated butanol and the color was developed at room temperature for 24 hours.

Displacement chromatograms were obtained as follows. The nitrogenous compounds in an alcohol extract from 600 g. of salmon muscle were adsorbed on a cation exchange resin and displaced with 0.075 *N* sodium hydroxide as described by Shewan (14). The resin used was Bio Rad AG50×4 and the fractions collected were 4 ml. in volume. The compounds present in each fraction were identified (14) and the results are shown diagrammatically in Fig. 1 in order that the relative amounts of the compounds with respect to one another can readily be seen.

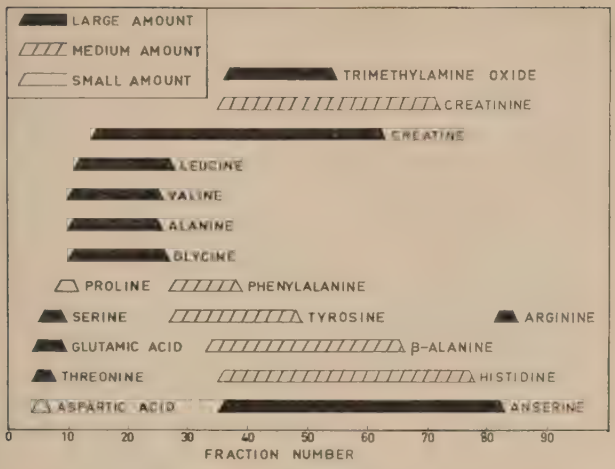

FIG. 1. Compounds displaced from cation exchange resin with sodium hydroxide.

Results and Discussion

The non-protein nitrogenous constituents of sockeye salmon muscle were investigated at three points during the migration of the fish up the Fraser River, namely at Lummi Island at the mouth of the river, at Lillooet 250 miles upstream, and at the Forfar Creek spawning grounds 753 miles upstream (3).

The muscle extracts from all fish of the same sex from each location were combined and aliquots were subjected to two-dimensional paper chromatography as described under methods. Thirteen ninhydrin spots were observed to be common to all chromatograms. Moreover the size of these spots relative to one another appeared to be constant from one chromatogram to another. In addition to the 13 spots already mentioned, a spot in the position of histidine was observed in chromatograms from both male and female fish at Lummi Island. This spot was not observed in chromatograms from fish of either sex at Lillooet or Forfar Creek.

The ninhydrin-reacting compounds were identified by their R_f values on the paper chromatograms, and further proof of their identity was obtained by employing displacement column chromatography. Figure 1 shows diagrammatically the displacement picture obtained with extracts from the muscle of male fish at Lummi Island. All the compounds identified on the two-dimensional paper chromatogram were found in the displacement chromatogram with the exception of taurine, which was not adsorbed by the resin in the column and therefore not displaced by the sodium hydroxide. The displacement picture shows, in addition, the presence of a trace of proline and the compounds creatine, creatinine, and trimethylamine oxide, which did not give a colored spot with ninhydrin. Fig. 1 also gives an indication of the relative amounts of the compounds in the muscle with respect to one another.

The fact that histidine was observed in chromatograms of muscle extracts of the fish at Lummi Island but not at other points on the river led to further investigations of that compound. Analyses showed that the histidine content of the muscle dropped sharply from Lummi Island to Lillooet and remained at a low level thereafter. These results are shown in Table I. In both male and female fish the histidine value decreased to about one fifth of the value at Lummi Island. At present no explanation can be given for this phenomenon.

TABLE I

THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF SOCKEYE SALMON MUSCLE AT DIFFERENT LOCATIONS ON THE MIGRATORY ROUTE

(All values are mg. nitrogen per 100 g. wet muscle)

Component	Sex	Lummi Island	Lillooet	Forfar Creek
α-Amino-N	M	94.8±4.7	88.4±2.3	94.4±5.5
	F	69.8±3.6	65.5±2.1	81.6±3.2
Volatile base-N	M	10.5±1.2	7.2±0.3	8.5±1.0
	F	9.3±0.7	7.9±1.2	11.4±0.6
TMO-N	M	5.4±0.3	5.4±0.8	5.3±0.4
	F	5.4±0.7	5.7±0.6	6.4±0.2
TM-N	M	<0.2	<0.2	<0.2
	F	<0.2	<0.2	<0.2
Creatinine-N	M	4.9±0.8	5.9±0.4	5.6±1.2
	F	3.7±0.4	3.7±0.3	3.1±0.5
Creatine-N	M	77.9±8.1	91.4±5.4	95.1±8.7
	F	95.5±11.8	114.4±2.8	127.0±3.6
Histidine-N	M	10.8±1.3	2.1±0.5	1.3±0.1
	F	15.1±0.8	2.7±0.4	3.1±0.1
Anserine-N*	M	24.8	31.2	30.2
	F	29.4	27.5	36.1

*These values were obtained using combined extracts from the four groups.

Table I gives the results of analyses on the salmon muscle. The values in the table are the average for four groups (3), plus or minus the standard error. These results indicate that muscle from male and female fish had the same non-protein nitrogenous constituents, and that these constituents were present in approximately the same amount in both sexes, except for creatine, which was present in somewhat larger amounts in the female, and α-amino nitrogen, which was found to a slightly greater extent in the male. The values obtained for these compounds were similar to those obtained (1) for king salmon. It is interesting to note that the dipeptide anserine was present in relatively large amounts in the muscle of both male and female fish. Little, if any, trimethylamine was present, but trimethylamine oxide was found in small quantities.

In comparison with histidine the amounts of the other constituents showed little change during the migration. Of these other compounds, creatine showed the greatest change. An increase in the level of this compound was observed during migration, especially with female fish. The α-amino nitrogen in both sexes appeared to decrease slightly from Lummi to Lillooet, and then to increase from Lillooet to Forfar Creek, but the only change which was

significant within the 1% confidence limits was that in the females between Lillooet and Forfar Creek. The volatile-base nitrogen followed a similar trend to the α-amino nitrogen.

It is seen from Table I that there was an over-all tendency for the non-protein nitrogenous constituents to increase during the journey of the female fish from Lillooet to Forfar Creek, and that this increase was much more noticeable than that observed in the male fish. However, Idler and Tsuyuki (3) have shown that the weight of the muscle in "standard fish" decreased during spawning migration, and that this loss in muscle weight was greater in the female sex. The figures obtained by Idler and Tsuyuki were employed in conjunction with the results in Table I to give an estimate of the "total amount" of non-protein constituents in the muscle. These results are given in Table II. Little or no increase in the constituents was observed from the Lillooet location to the Forfar Creek location. In other words, the increase in the concentration of the muscle constituents was balanced by the decrease in the weight of the muscle. Therefore, although the concentration of the constituents increased during the latter stages of the migratory journey, this was brought about by a decrease in the amount of flesh rather than in increase in the amount of the constituents. The results in Table II show that the main changes in the "total amounts" of the constituents during migration were small decreases in the amounts of α-amino nitrogen and volatile bases and a large decrease in histidine during the early part of the migratory journey.

TABLE II

THE AMOUNT OF NON-PROTEIN NITROGENOUS CONSTITUENTS IN THE MUSCLE OF THE "STANDARD FISH"

(All values are mg. nitrogen per fish)

Component	Sex	Location		
		Lummi Island	Lillooet	Forfar Creek
α-Amino-N	M	1391	1151	1231
	F	931	743	762
Volatile base-N	M	154	94	111
	F	124	90	106
TMO-N	M	79	70	69
	F	72	65	60
Creatinine-N	M	72	77	73
	F	50	42	29
Creatine-N	M	1143	1190	1240
	F	1274	1298	1186
Histidine-N	M	158	27	17
	F	202	31	29
Anserine-N	M	364	406	394
	F	392	312	337

Acknowledgments

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The Keeping Time of Wrapped Fillets Treated with an Antibiotic

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IN a previous issue (June, 1958), *Canadian Fisherman* published an article entitled "Should we pre-wrap fresh fillets in consumer-size packets?". This pointed out the advantages and disadvantages of wrapping fillets in transparent, air-tight wrappers. From the standpoint of sanitation and customer appeal there is an advantage to the wrappers. On the other hand, it was shown that unwrapped fillets stored on ice had a longer keeping time than those in air-tight wrappers.

The article also stated that "in actual practice the keeping time of the wrapped fillets could be easily extended by the use of a suitable antibiotic or some other acceptable preservative". The question is: by how many days could the keeping time be extended? Once again we let the fish answer for themselves. This is how it was done. We found a commercial fish plant that was normally using an antibiotic as a preservative for their fresh fillets. They used "Acronize", which is a trade name for chlortetracycline; and they used a dip that contained approximately 10 ppm. of this antibiotic. From this firm we obtained 100 fresh untreated haddock fillets and 100 similar fillets that had been dipped in the preservative at the plant.

As in the experiments described in the previous article, half of these fillets were stored unwrapped in ice and the

TABLE I.—The development of off-odours in wrapped and unwrapped haddock fillets. Half the fillets were dipped in an antibiotic preservative at the fish plant (the dip contained 10 ppm. chlortetracycline, sold under the trade name of "Acronize"), and the remainder were left untreated.

	FILLET ODOUR			
	Unwrapped fillets on ice		Wrapped fillets in cool storage	
Days in storage	Acronized	Untreated	Acronized	Untreated
3	No off-odours	No off-odours	No off-odours	No off-odours
4	"	"	"	"
5	"	"	"	Slightly sour
7	"	"	None to slight off-odours	Sour
9	"	"	Slightly sour	Putrid
12	"	Slightly sour	"	"

TABLE II.—Trimethylamine is one of the spoilage products that accumulates as fish muscle deteriorates and is frequently used as a measure of the amount of spoilage that has taken place in sea fish such as cod or haddock.

	TRIMETHYLAMINE VALUES*			
	Unwrapped fillets		Wrapped fillets	
Days in storage	Acronized	Untreated	Acronized	Untreated
0	0.18	0.15	0.20	0.18
3	0.22	0.38	0.30	0.70
5	0.20	0.50	0.45	3.20
7	0.30	0.52	0.48	11.70
9	0.21	0.61	1.74	15.54
10	0.28	1.29	1.20	15.13
12	0.65	2.97	2.04	15.64

*mg. of trimethylamine nitrogen per 100 g. of fillets.

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other half were wrapped in waxed paper and stored in watertight aluminum containers that were buried in ice. In both cases the temperature of the fillets remained fairly constant at 32°F. throughout the test period. Periodically a number of fillets was withdrawn from each lot, carefully examined for indications of spoilage and tested for trimethylamine content.

Results of Tests — The results are summarized in the accompanying tables. The wrapped, untreated fillets showed the first signs of spoilage at 5 days and were inedible at 10 days. The wrapped, "Acronized" fillets began to spoil at 8 or 9 days and were approaching the inedible state at 12 days. The untreated, unwrapped fillets on ice were also quite acceptable up to 9 days, but had only slightly sour odours at 10 days. The "Acronized" fillets stored in ice were edible at 12 days and probably would have been edible for several more days if the experiment had been continued.

This does not mean that all wrapped and "Acronized" cod or haddock fillets held at 32°F. will necessarily keep in a good state of preservation for 9 or 10 days. In actual practice some will keep longer and some will keep less.

Tests with various lots of "Acronized" commercial cod and haddock fillets made at this Station have shown keeping times ranging all the way from 8 to 22 days at 32°F., with most of them in the range of 10 to 14 days. The reason for these differences in keeping time is the initial quality of the fish at the time it is cut and the subsequent care in handling and chilling. Most of these tests have shown that the antibiotic just about doubles the keeping time (using 10 ppm. dip for 15 seconds)*.

It has been suggested that if the air was removed from the plastic packages containing the fillets they might last longer. In tests made here with Acronized fillets in plastic containers with and without air removal, it was found that the air removal did not improve the keeping time.

It has also been suggested if the antibiotic dip was made up with 15% brine instead of plain water, the keeping time would be increased. This has also been tried out with commercial fillets. It was shown that the brine might retard the initial spoilage for about one extra day, but once spoilage got under way, the brine-dipped fillets reached the inedible stage before those that were unsalted.

Significance — What is the commercial significance of the results of these tests for those who are interested in pre-wrapping fillets in consumer-size packages? In the first place, if fresh untreated fillets are to be wrapped, they will probably remain in a very good state of preservation for 4 or 5 days. If they are to be shipped to the central Canadian markets or to the middle west, that does not leave them with much of a "shelf life". The wrapped fillets that were Acronized had a "safe" period of 9 or 10 days. This would give 1½ or 2 days to get the fish onto the market and then a shelf life of approximately one week.

**If sodium nitrite is used as a preservative dip in place of the antibiotic, the keeping time of the treated fillets will be cut down by several days.*

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A Direct-Current Electrofishing Apparatus Using Separate Excitation

by

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In order to determine the standing crops of Atlantic salmon, brook trout, and eels in the Little Codroy River, Newfoundland, a direct-current electrofishing apparatus using separate excitation has recently been devised. Separate excitation is a simple and practical method for getting excellent output voltage control. It is also a safer and more economical means of producing power for electrofishing. The purpose of this paper is primarily to describe the unit and report briefly on some results of its use.

The electrofishing apparatus consisting of engine, generators, control panel, cable and reel, positive fishing electrode, and negative ground electrode, is shown in Figure 1A. The source of power is a 6.5 h.p. Briggs and Stratton air-cooled, gasoline engine. An engine of this size was selected in order to take advantage of its relatively low r.p.m. characteristics (2,400 r.p.m.). The generators are belt driven (Figure 1B), with the exciter generator turning at 1,440 r.p.m., and the main generator at 1,250 r.p.m.

The wiring diagram for the electrofishing apparatus is shown in Figure 2. The main generator (Figure 1C) is a rebuilt, separately excited, direct-current unit mounted in an "Imperial Electric" frame. The armature is capable of producing a maximum unloaded potential of 675 volts at 3 amperes continuous service, or 4 amperes intermittent service. The resistance of the armature is 4 ohms. The field is wound for separate 12-volt excitation with an external field rheostat mounted in the control panel for output control. The resistance of the field is 36 ohms. Interpole windings are used to improve voltage regulation under varying load conditions.

The exciter generator (Figure 1D) is a Lucas 12-volt, shunt-wound, direct-current generator. There are three reasons for using separate excitation. First, excellent voltage control can be obtained using separate excitation (along with interpole windings) because field troubles from variable voltage and load are minimized. Separate excitation means that the main generator has a low voltage separately excited field (i.e., 12 volts, as compared with 675 volts if the generator were self-excited). This minimizes the danger of insulation breakdown and flashover in the damp conditions existing beside rivers. Second, safety is a feature of separate excitation because 12 volts are used to control 675 volts. There are actually two safety factors involved: (*a*) the 12 volts separate excitation, and (*b*) the relay which is mounted in the control panel. The 12-volt generator is the source of power for the relay. The switch in the handle of the positive electrode (Figure 2) controls the relay, and the relay, in turn, controls the

FRB No. 541

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field of the main generator. Thus there is just enough low voltage current flowing through the electrode switch to operate the relay. Third, because the resistors, relay, and switches are in the 12-volt circuit they are low priced and easily obtainable.

The control panel (Figure 1E) consisting of field rheostat, voltmeter, ammeter, 12-volt relay, and main switch, is mounted in a standard steel junction box. The

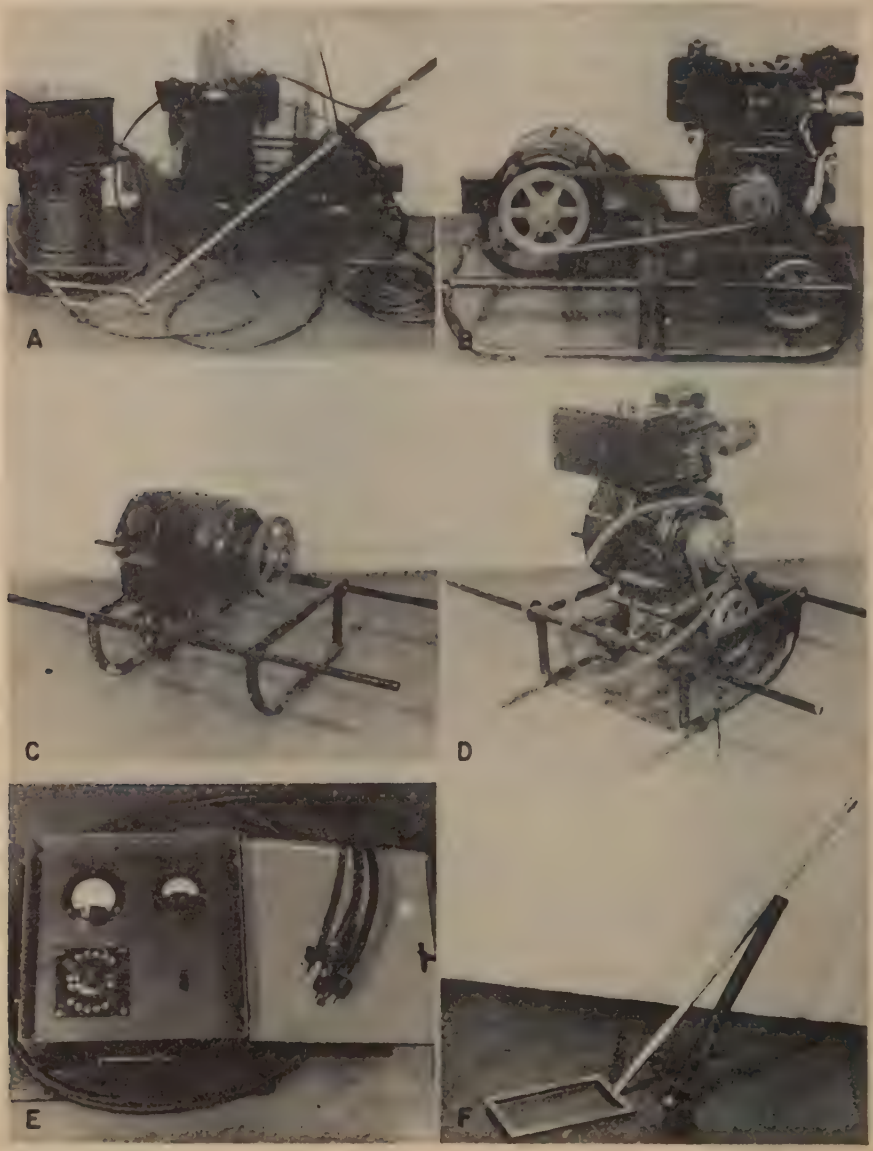

Figure 1.—Direct-current electrofishing apparatus.

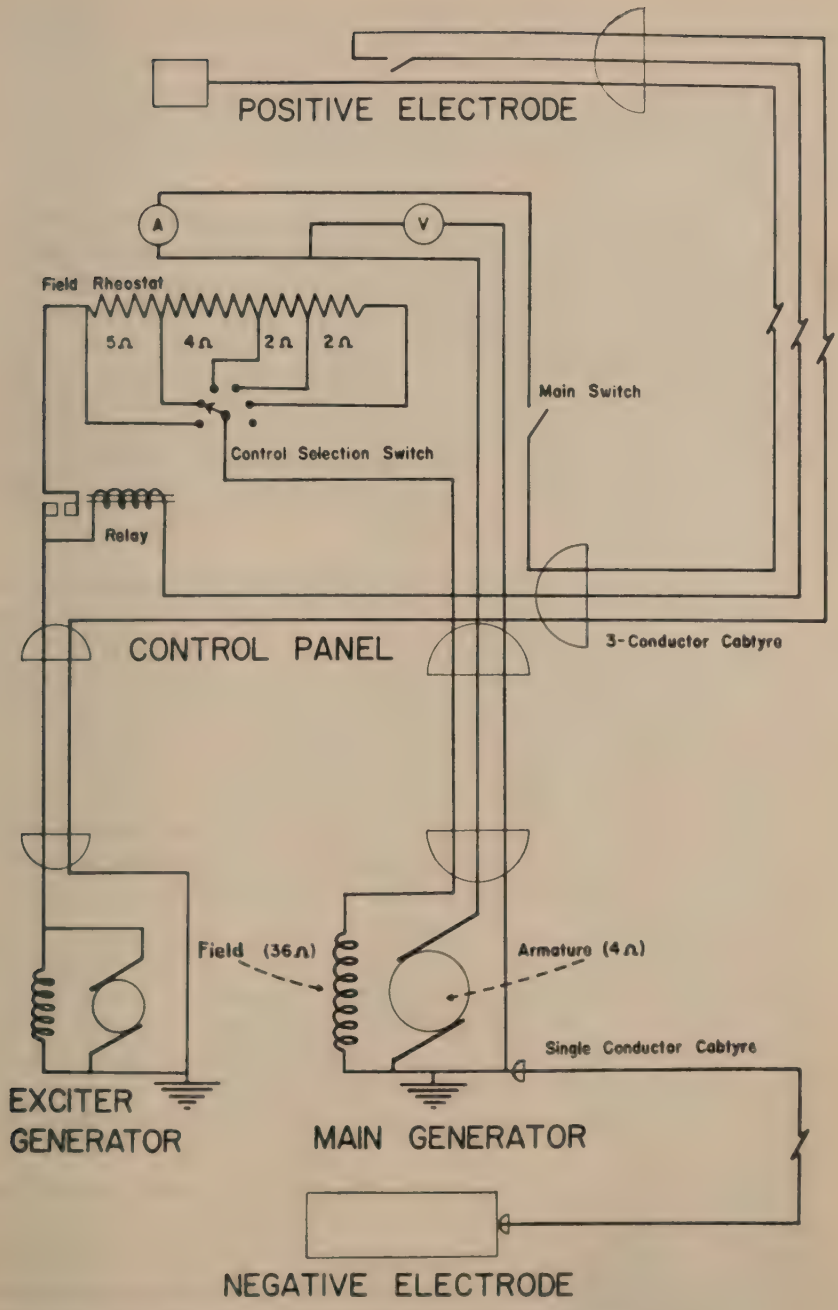

Figure 2.—Schematic wiring diagram of direct-current electrofishing apparatus.

control switch on the 13-ohm field rheostat allows the selection of any one of 5 output voltages (unloaded values): 200, 300, 400, 520, and 675. The variable output voltage is a useful feature for it allows the selection of the voltage best suited to the conductivity of the water, as well as the size and species of fish being collected. The control panel is separate from the engine and generators, the circuits being completed by means of two short cables from the control panel plugged into polarized receptacles on each generator.

The engine and generators are bolted to a light weight tubular steel (bicycle-type) frame made in two sections, one carrying the engine and exciter generator, and the other the main generator. The weight of the engine and exciter section is 168 pounds, and the weight of the main generator section is 144 pounds. The two sections are carried separately (Figures 1C and 1D), but are bolted together when the unit is in operation (Figures 1A and 1B). The main generator can be moved back and forth on its frame to allow for belt adjustment. As suggested by Smith and Elson (1950), the engine and generators are mounted on two frames for convenience in handling because of their weight. However, an overall improvement in portability has not resulted because of their bulk and also because of the weight of some of the accessory gear (such as the barrier nets). Hence it is possible to use this equipment only in the areas of the river which are accessible by vehicle.

The cable reel (Figure 1A) is a metal flanged drum which carries 300 feet of 3-conductor, number 14, cabtyre cable. One end of the cable is plugged into a polarized receptacle on the control panel, and the other end into a polarized receptacle on the handle of the positive fishing electrode. In practice, the reel is mounted on a horizontal axle, which is supported by a stand made of steel pipe, and the approximate amount of cable required is pulled from the reel and stretched along the stream bank. The man operating the fishing electrode drags the cable behind him. The negative pole of the main generator is grounded by connecting it through 100 feet of single-conductor, number 14, cabtyre to a 3- by 10-foot piece of copper hardware cloth which is set on the stream bottom and covered with rock or gravel. The single-conductor cable is also carried on the reel. The total weight of the reel and both cables is 114 pounds.

The positive fishing electrode (Figure 1F) consists of a straight piece of $\frac{5}{8}$ inch copper tubing, on the distal end of which is a rectangular piece of $\frac{1}{2}$ inch copper tubing, size 8 by 11 inches. The proximal end of the electrode is attached to a tubular insulated handle by means of a brass coupling. There is a sliding contact switch on the handle which indirectly controls the main generator through the relay, hence the high potential will not be produced unless both the electrode switch and relay are closed. All members of the electrofishing crew wear electrician's rubber gloves and rubber chest waders.

A Jeep and 2 wheel, $\frac{1}{2}$-ton trailer are used to transport the crew, apparatus, and accessory equipment.

The unit has been used under a wide variety of conditions on the Little Codroy River, and has proven highly satisfactory. It has, for example, been used in both winter and summer in water ranging from 2.8°C. to 19.2°C. It has been used in various parts of the drainage basin in water the resistivity of which has varied from 19,800

to 42,200 ohms per centimeter cube ($\Omega/cm.^3$). The positive fishing electrode has been fished up to 400 feet from the negative ground electrode with no apparent decrease in efficiency.

The total numbers of fish captured at the various sampling stations are shown in Table I. Because this work is in its preliminary stages, a more complete analysis is not given. The data are included only to indicate that the apparatus has been successfully used to catch Atlantic salmon parr, brook trout, and eels under a variety of conditions. The method of blocking off the sample areas with barrier nets, and the fishing procedure used at each station were much the same as that described by Godfrey (1956).

Observations on the behaviour of Atlantic salmon parr, brook trout, and eels in the direct-current field parallel those of Godfrey. As reported by Saunders and Smith (1954), it was found that collection of fish was easiest when the generator voltage was adjusted so that the fish could be held at the positive fishing electrode in a condition of partial paralysis. The potential was around 300-375 volts. In general, voltages around 300 were found to be more satisfactory in warm water, whereas voltages around 375 were more satisfactory in cold water. This complements the findings of Smith and Elson.

Table I

Numbers of fish taken at each of the sampling areas, Little Codroy River, 1956.

Station Number	Atlantic Salmon Parr		Brook Trout	Eels
	Under-yearlings	Yearlings and Older		
2	38	35	0	31
3	26	57	1	46
5	31	47	1	27
6	63	30	23	53
8	237	22	10	5
9	1	3	0	28
16	92	78	19	59
16a	110	74	13	5
17	0	39	4	32
18	56	47	15	18
19	29	89	2	66

In connection with voltage adjustment, it was observed that the lower the resistivity of the water, the lower the voltage produced, and the greater the current drawn, at any one setting of the control selection switch on the field rheostat. For example, when the control selection switch was set so that the main generator was

producing 650-675 volts (no load), under load in water of 19,800 Ω/cm.^{3} resistivity it was producing 330 volts at 1.7 amperes, and in water of 42,200 Ω/cm.^{3} resistivity it was producing 600 volts at 0.9 amperes. The effect of water temperature and water resistivity on voltage adjustment were also observed in a general way. At any given resistivity, the higher the water temperature, the lower the voltage produced, and the higher the current drawn. For example, with the control selection switch set as above, the main generator produced 500 volts at 1.2 amperes in water of 40,200 Ω/cm.^{3} and 15.3°C., whereas it produced 600 volts at 0.9 amperes in water of 42,200 Ω/cm.^{3} and 4.4.°C.

Some immediate mortality was observed in two instances when fishing in rapid water over rubble bottom when dislodged and shocked parr drifted downstream into the lower barrier net and were held by the pressure of the water and by the accumulation of debris on the net. This occurred early in the season when the apparatus and its attendant techniques were new to the crew. As the season progressed and the crew became more proficient, there was no further immediate mortality.

Shortly after the unit was completed in January 1956, it was tested in the Salmonier River, St. Mary's Bay, Newfoundland. Approximately 160 Atlantic salmon and brown trout parr were collected. These fish were brought back to the Biological Station and held for observation in wooden troughs and glass aquaria for a period of one month. There were no mortalities during this period. Accordingly, delayed mortality has been assumed to be not significant, and it has further been assumed that fish which recovered immediately from the effects of the electroshock were uninjured. Similar conclusions have been drawn by Smith and Elson.

Acknowledgements

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NITROGEN EXCRETION IN SOME MARINE TELEOSTS[1]

J. D. Wood

Abstract

The nitrogen-containing compounds excreted by the sculpin, starry flounder, and blue sea-perch were investigated. Approximately four-fifths of the nitrogenous material was excreted via the gills. Ammonia and urea were the main components of the excreta in all three species. The amount excreted in 24 hours varied greatly between fish of any one species, but the percentage composition was reasonably constant. A greater variation in the composition occurred between species. Trimethylamine oxide was present in only small amounts in the total excreta. It was concluded from this that the compound was not an important end product of protein metabolism.

Introduction

The nitrogenous material excreted by the animal body is of interest because it reveals the end products of protein metabolism within the animal. In the fish kingdom the excreta of marine teleosts have been studied extensively and particular attention has been paid to trimethylamine oxide (TMO). This compound was first isolated from fish by Suwa (1), who obtained it from dogfish muscle. It has since been shown to occur extensively in different species throughout the marine world (2). Grollman (3) reported that 37% of the total non-protein nitrogen in the urine of *Lophius piscatorius* was present as TMO-N. This was confirmed by Grafflin and Gould (4). These workers found that sculpin urine likewise was rich in TMO but that flounder urine contained very little of the compound. Urea-N accounted for approximately 15% of the total N in the urines of sculpin and flounder, but little urea was found in urine of *Lophius piscatorius*. Ammonia was present only in small amounts in the urines of all three species.

The physiological role of TMO has been debated for a long time and a review of the different theories is given by Shewan (2). Among the theories mentioned are, (*a*) that TMO is the non-toxic end product of protein metabolism, (*b*) that TMO plays an important part in osmoregulation, and (*c*) that TMO is of exogenous origin, being obtained from the food of the fish.

Smith (5) found that fresh-water teleosts excreted 80% to 90% of the nitrogenous material via the gills and the remainder by the kidneys. Therefore, the percentage composition of the total excreta could be quite different

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F.R.B. No. 542

from that of the urine since the composition of the total excreta will be influenced mostly by the material eliminated via the gills. If marine teleosts likewise eliminate most of the nitrogenous compounds via the gills, then TMO may not have the significance that the composition of the urine suggests. Delaunay (6) measured the total excreta of the sole and he found that ammonia was the major nitrogenous constituent. This was a very different composition from that found for the urines previously mentioned. Whether the variations in composition were due to differences between species or between total and urinary excretions was not investigated. Neither did Delaunay determine TMO in the excreta of the sole.

A certain confusion seems to have arisen in textbooks concerning the nitrogen excretion of marine teleosts. Prosser (7) reports the results of Delaunay and of Grafflin and Gould but does not differentiate between urinary excretion and total excretion. Baldwin (8) states that one-third of the total excreta of marine teleosts is in the form of TMO, and refers to H. W. Smith's results. However, the latter employed fresh-water teleosts and he did not determine TMO. No report of similar experiments on marine teleosts by Smith could be found in the literature.

The present experiments were carried out to determine the composition of the excreta from different species of marine teleosts with particular reference to TMO.

Materials and Methods

Fish

The species employed in the experiments were the sculpin, *Leptocottus armatus*; the starry flounder, *Platichthys stellatus*; and the blue sea-perch, *Taeniotoca lateralis*. The fish were caught in the Straits of Georgia, British Columbia, and kept in aquaria until required. During captivity they were fed pieces of lingcod muscle.

Collection of Excreta

The fish specimen under consideration was placed in a tank slightly larger than itself and sufficient sea water was added to cover the fish. This usually required between 1 and 2 liters of water. The water was well aerated and was kept at 12° C by placing the tank in a constant flow of cold water. After 24 hours the fish was removed from the tank and the water acidified with a small amount of HCl and taken down to dryness *in vacuo* at 45° C. The non-protein nitrogenous compounds were largely separated from the inorganic salts with four successive extractions of 80% ethanol. The total volume of ethanol used was one-seventh of the original volume of the sea water. The ethanol extracts were combined, filtered, and taken down to dryess *in vacuo* at 45° C. The residue was redissolved in water to give a final volume of 50 ml.

This extraction procedure yielded 97% recovery when applied to known amounts of ammonia, urea, trimethylamine (TM), TMO, and creatine dissolved in sea water.

Analyses

The total N was estimated using the micro-Kjeldahl method as adapted by Ma and Zuazaga (9).

The ammonia-N, TM-N, and TMO-N were determined by the method of Ronold and Jakobsen (10). In this method the ammonia-N may include some methylamine-N and dimethylamine-N. The presence of the latter two compounds can be detected using the Conway microdiffusion cell. If the outer compartment is buffered at pH 9.5 with borate, then the amines diffuse into the center compartment much more slowly than ammonia. After 5 hours at 20° C., 94% of the ammonia had diffused into the center well compared with 9% of the methylamine and 8% of the dimethylamine. The material from sculpin excreta diffused rapidly, 91% being found in the center well after a period of 5 hours. It was therefore concluded that the ammonia determined in the excreta was only slightly, if at all, contaminated with the amines.

Urea-N was measured by the methods of Fearon (11) and Koritz and Cohen (12), and good agreement was obtained between the two methods. These methods estimate compounds containing a ureide grouping, but of all the compounds giving a positive test only urea gives a characteristic bright yellow color using the method of Fearon. The excreta of the fish gave this characteristic color, but to check the identity of the ureide-containing compound more accurately, the absorption spectra of the colored solutions were measured. Table I shows the wavelength of maximum absorption and the

TABLE I

LIGHT ABSORPTION OF COMPOUNDS CONTAINING A UREIDE GROUPING

	Wavelength of maximum absorption (mμ)	Absorption at 484 mμ / Absorption at 495 mμ
Urea	484	1.34
Methyl urea	492	0.93
Citrulline	496	0.78
Sculpin excreta	484	1.33

ratio of the absorptions at 484 mμ and 495 mμ for different compounds and for sculpin excreta. These results show that the material in the sculpin excreta is mostly, if not entirely, urea. The method of Koritz and Cohen was the one employed during the experiments because of its greater sensitivity. The authors of this method point out that biological material sometimes increased slightly the optical density produced by a given amount of compound. In order to eliminate this possible source of error, urea standards were prepared using the excreta solution as solvent. The optical density due to the added urea was then obtained by difference.

Creatine-N and creatinine-N were estimated by the method of Bonsnes and Taussky (13). Prior to these determinations 2 *N* NaOH was added

drop by drop until no further precipitation of inorganic material occurred. The precipitate was removed by centrifugation, resuspended in 0.5 *N* NaOH, and again centrifuged. The two supernatant fractions were combined and after neutralizing with HCl they were ready for use in the estimations. The method of extraction of the excreta from sea water was found to change a small part of the creatine to creatinine. Therefore the creatine-N and creatinine-N values were combined in the results presented here.

Results

An experiment was carried out similar to that described by Smith (5) to determine the proportions of the nitrogenous material excreted by the gills and by the kidneys, respectively. A sculpin was employed as the experimental fish and it was found that 77% of the total N was excreted via the gills.

The excreta from seven sculpins, two starry flounder, and a blue sea-perch were investigated and the results are shown in Table II. The total amount excreted varied considerably between fish of the same species, but in all cases the major components of the excreta were ammonia and urea. The predominance of these compounds in the excreta was observed in all three species of fish investigated.

TABLE II

NITROGEN EXCRETION IN MARINE TELEOSTS

	Sculpin							Starry flounder		Blue sea-perch
	1	2	3	4	5	6*	7*	1	2	1
Weight, g	165	182	191	391	151	245	304	335	310	360
Total N	8.31	7.45	24.88	24.38	6.04	16.22	18.50	22.46	24.52	10.71
Ammonia-N	4.37	4.66	16.77	17.64	4.02	10.11	10.23	19.37	19.97	5.14
Urea-N	1.87	1.53	6.15	4.22	1.02	3.90	3.59	2.58	2.96	4.08
Trimethylamine-N	0.00	0.10	0.39	0.33	0.11	0.46	0.36	0.25	0.05	0.03
Trimethylamine oxide-N	0.18	0.00	0.33	0.93	0.05	0.00	0.04	0.12	0.00	0.04
Creatine-N + creatinine-N	0.03	—	0.23	0.17	0.03	0.18	0.26	0.06	0.30	0.00

NOTE: All N values in the table are given as mg.
*These fish were newly caught and had not been kept in aquaria prior to collection of excreta.

When these results are converted to percentages of the total N excreted, Table III, it is observed that the ammonia and urea contents of the excreta remained reasonably constant within any one species, although the size of the fish varied considerably. Moreover, the newly caught fish showed the same percentage composition of ammonia and urea as did those which had lived for a considerable length of time in the aquarium. The same constancy of composition, however, did not hold from species to species. The starry flounder had a greater percentage of ammonia and less urea than the sculpin, and the blue sea-perch had less ammonia and more urea.

The average undetermined-N values in the excreta of the sculpin, starry flounder, and blue sea-perch were 13%, 3%, and 13% respectively. The

TABLE III

PERCENTAGE COMPOSITION OF EXCRETA FROM MARINE TELEOSTS

	Sculpin							Starry flounder		Blue sea-perch
	1	2	3	4	5	6	7	1	2	1
Ammonia-N	52.6	62.6	67.4	72.4	66.6	62.3	55.3	86.2	81.4	48.0
Urea-N	22.5	20.5	24.7	17.3	16.9	24.0	19.4	11.5	12.1	38.1
Trimethylamine-N	0.0	1.3	1.6	1.4	1.8	2.8	1.9	1.1	0.2	0.3
Trimethylamine oxide-N	2.2	0.0	1.3	3.8	0.8	0.0	0.2	0.5	0.0	0.4
Creatine-N + creatinine-N	0.4	—	0.9	0.7	0.5	1.1	1.4	0.3	0.1	0.0
Undetermined N	22.3	15.6	4.1	4.4	13.4	9.8	21.8	0.4	6.2	13.2

undetermined N was investigated in the sculpin excreta. Tests were made for uric acid and amino acids but neither was detected.

The effects of bacterial action on the excreta were investigated. A sculpin was placed in a tank of water and after 24 hours the fish was removed and a sample of water analyzed for ammonia and urea. The remaining water was left in the tank for a further 24 hours under the same conditions of temperature and aeration as previously. At the end of this time a sample of the water was taken and the ammonia and urea contents compared with the first sample, Table IV. Little change is observed in the contents of the two samples.

TABLE IV

EFFECT OF BACTERIA ON EXCRETA

	Ammonia-N	Urea-N
Water previous to standing for 24 hours	10.95	2.32
Water after standing for 24 hours	10.62	2.25

NOTE: All figures are mg nitrogen per liter water.

TABLE V

CHANGES IN NITROGENOUS COMPOUNDS IN SEA WATER DUE TO THE PRESENCE OF A SCULPIN

Sample	Ammonia -N	Trimethyl-amine-N	Trimethyl-amine oxide -N	Urea -N	Creatine-N + creatinine-N
Water initially	0.6	6.6	8.0	7.2	8.2
Water after containing sculpin for 24 hours	10.4	6.5	8.2	9.1	8.6
Excreted by sculpin in previous 24 hours	7.7	0.0	0.1	1.7	0.2

NOTE: All figures are mg nitrogen.

The possibility was considered that bacteria on the surface of the fish might act on the excreta. This was investigated by placing a sculpin in a solution of sea water containing known amounts of ammonia, urea, TM, TMO, and creatine. At the end of 24 hours the water was analyzed for these compounds,

and the amounts compared with the original quantities. The results are shown in Table V together with the nitrogenous material excreted by the sculpin during the 24 hours previous to the experiment. The changes in the amounts of the original compounds are consistent with what might be expected when the excretion of the sculpin in 24 hours is taken into consideration.

It appears, therefore, that the excreta of the fish were not attacked by bacteria under the conditions being used for the experiments.

Discussion

Although generalizations about marine teleosts cannot be made on the basis of results from only three species, the results reported here suggest that ammonia and urea are the main nitrogen-containing compounds excreted by marine teleosts. Together these compounds account for 75% to 98% of the total N. Of the two compounds, ammonia is present in the greater quantities.

TMO accounts for approximately one-third of the total N in the urine of some species (3, 4). However, the amount of nitrogenous material excreted in the urine is small compared with that excreted by the gills. It is not too surprising therefore to find that the proportion of TMO in the total excreta is relatively small. The low percentage of the compound in the excreta indicates that the compound is not an important non-toxic end product of protein metabolism in marine teleosts as previously suggested (8, 14). The low and rather varied values are more in accordance with the theory of Norris and Benoit (15) that TMO in marine teleosts is obtained directly from the food, i.e. it is of exogenous origin.

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THE IDENTITY OF THE SPECIES OF LEPEOPHTHEIRUS (COPEPODA) PARASITIC ON PACIFIC SALMON (GENUS ONCORHYNCHUS) AND ATLANTIC SALMON (SALMO SALAR)[1]

L. MARGOLIS

Abstract

A review of existing descriptions of *Lepeophtheirus* from salmonids, based on specimens collected mainly from *Salmo salar* in the European and North American Atlantic and from *Oncorhynchus* spp. in the Asiatic and North American Pacific, coupled with observations by the author on material from *S. salar* from England and from *Oncorhynchus* spp. from a wide range of localities in the North Pacific, suggest that *L. salmonis* (Krøyer, 1838) is the only species found on salmonids from both oceans. The differentiation of *L. uenoi* Yamaguti, 1939 as a distinct species on Pacific salmon seems to be the result of incorrect or inadequate early descriptions of *L. salmonis*.

Lepeophtheirus salmonis (Krøyer, 1838) was originally described from the Atlantic salmon, *Salmo salar*, and is a common external parasite of this salmon in both European and North American Atlantic and adjacent waters. Also it has been recorded many times from five species of Pacific salmon of the genus *Oncorhynchus* in North American and Asiatic Pacific waters. Other anadromous salmonids of the genera *Salmo* and *Salvelinus* also have been reported as hosts. The apparent favorite location of this parasite is in the perianal region of the fish but it may be found on any part of the body or occasionally even on the gills.

Until 1939 the species of *Lepeophtheirus* on Pacific salmonids was considered to be identical with the Atlantic species.[2] Since then Japanese workers have described the species found on *Oncorhynchus* from western Pacific areas as distinct, under the name of *Lepeophtheirus uenoi*. Yamaguti (10) first described this species from specimens collected from *Oncorhynchus gorbuscha* from Hokkaido. In comparing his material with the descriptions of *L. salmonis* as provided by Wilson (9) and Scott and Scott (6) he noted the following differences: (1) "the basal segment of the second antenna has a stout spiniform process on the posterior margin and" (2) "the second segment of the fourth leg is provided at the tip with a well-curved claw" in *L. uenoi*, both of which are lacking in *L. salmonis* according to Wilson (9) and

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[2]A second species of *Lepeophtheirus*, *L. pacificus* (Gissler, 1883) Wilson, 1905 was described (1, 9) from a salmon (not specifically identified, but probably *Oncorhynchus nerka*) taken in Puget Sound, Washington. Wilson (9) expressed the opinion that on the basis of the original description and illustrations, *L. pacificus* is distinct from *L. salmonis*. Gissler's (1) description lacks many details but nevertheless, contrary to Wilson's opinion, there is a great deal of similarity between Gissler's illustrations of appendages of *L. pacificus* and those of *L. salmonis*. In certain aspects Wilson's illustrations are inaccurate reproductions of Gissler's originals. *L. pacificus* has not been collected again and, according to Wilson (9), the type specimens apparently have been lost. The status of this species must be considered doubtful, but it is not unlikely that it is a synonym of *L. salmonis*.

the Scotts (6). Shiino (7) described *L. uenoi* from *O. keta* taken near the Kurile Islands, and in addition to the differentiating characters cited by Yamaguti, he added the following: "the terminal joint of the exopodite of the 2nd pereiopod bears a simple short spine outside the group of much longer plumose spines in the Atlantic species" (i.e. *L. salmonis*), "while 2 of such are discovered in the Pacific one" (i.e. *L. uenoi*).

Wilson (9), in his description of *L. salmonis*, stated that the basal joint of the second antenna lacks a spine on its posterior margin and that the second joint of the fourth leg has an enlarged tip, with rough papillary elevations, but without a spine. He did not describe the second leg but his illustrations indicate that there is only one small simple spine outside the group of plumose spines on the terminal segment of the exopod of this leg. Scott and Scott (6) wrote that the outer distal angle of the second joint (first joint of the ramus) of the fourth leg is in the form of a blunt knob covered with microscopic bristles. Their illustration of this leg does not show a spine on the second joint. In an earlier paper, the senior author (Scott (5)) remarked that the second joint of the fourth leg "is apparently not provided with a seta in this species, but is simply rounded". His illustration of the fourth leg shows the semicircular flange but no spine on the second segment. The Scotts (5, 6) did not describe or illustrate the basal segment of the second antenna or the second leg.

The collections examined by Wilson (9) came from a variety of sources and from several species of salmonids, including *S. salar* from England and the North American Atlantic coast, and from *Oncorhynchus* spp. caught in Alaska. Although Wilson did not state precisely upon which lot of specimens his description is based, he commented that the specimens from *S. salar* from England were used as the basis for identification of the North American material.

The descriptions by the Scotts (5, 6) were based on British specimens collected from *S. salar* (and possibly *Salmo trutta*).

In redescribing *L. uenoi*, Shiino (7) drew attention to White's (8) statements that specimens of *L. salmonis* taken from *S. salar* of the Canadian Atlantic coast differed from Wilson's (9) and Scott and Scott's (6) description in the possession of a spine on the second joint of the fourth leg. Shiino suggested that White's specimens might actually be *L. uenoi*.

Shiino, as well as Yamaguti (10), apparently neglected descriptions of *L. salmonis* published prior to that of Wilson (9) and also overlooked an important description of *L. salmonis* provided by Gurney (2). Although most of the older descriptions of *L. salmonis* lack many details pertaining to the structure of the appendages, Krøyer (4) had noted the presence of a very small curved thorn or claw on the second joint of the fourth leg (sixth leg of Krøyer) of specimens probably taken from *S. salar* in Denmark. Gurney based his account of the species on material collected from *S. salar* in Great Britain. With reference to the specific characters attributed to *L. uenoi*, he had written of *L. salmonis:* "Wilson states that the basal segment" (of

the second antenna) "lacks the spine on the posterior margin but it is present in my specimens." He further described the terminal segment of the exopod of the second leg "with 5 feathered setae and 3 outer spines, of which the distal one is very large." This distal spine actually bears bristles on one side, as seen in Gurney's illustration, thus leaving only two non-plumose small spines as described for *L. uenoi*. His illustration of this structure is in perfect agreement with those of Yamaguti and Shiino for *L. uenoi*. In spite of Gurney's statement that segment 2 of the fourth leg is without a spine, his figure of this leg (Fig. 1967, p. 333) clearly indicates a small curved spine on the outer distal angle, which is supposed to be characteristic of *L. uenoi*. Consequently Gurney's *L. salmonis* and *L. uenoi* are apparently identical.

Detailed illustrations of a female specimen of *L. salmonis* taken from *Leuciscus brandti* (an unusual host), recently published by Gusev (3), demonstrate the presence of the characteristics of the second antennae and second and fourth pereipods attributed by Japanese workers to *L. uenoi*. Basing his opinion on a re-examination of specimens from *S. salar* from the White Sea and from *O. gorbuscha* from the Okhotsk Sea, contained in the collection of the Zoological Institute of the Academy of Sciences of the U.S.S.R., Gusev concluded that *L. uenoi* was probably a synonym of *L. salmonis*.

Through the courtesy of Mr. R. U. Gooding, formerly of the Marine Laboratory, Plymouth, England, I have been able to examine a collection of *Lepeophtheirus* taken from *S. salar* from the River Tamar, England. None of this material agrees with the concept of *L. salmonis* as published by Wilson (9) and Scott and Scott (6), but all specimens fit Gurney's (2) account or illustrations of *L. salmonis*. I have also had the opportunity to collect and examine numerous specimens of *Lepeophtheirus* from five species of Pacific salmon, particularly *O. nerka* and *O. gorbuscha*, taken from many localities along the North American coast and from waters close to Asia, and from *Salmo clarki* from the British Columbia coast. All of these Pacific collections have been found to be morphologically identical with the specimens from *S. salar* which I have examined.

The descriptions and/or illustrations of Krøyer (4), Gurney (2), Yamaguti (10), White (8), Gusev (3), and Shiino (7), and personal observations of *Lepeophtheirus* from *S. salar* from European and North American Atlantic seas and from *Oncorhynchus* spp. from Asiatic and North American Pacific waters, therefore, suggest that only one species is involved and that its name should be *L. salmonis*, with *L. uenoi* as a synonym. It seems likely that errors or omissions in Wilson's and the Scotts' descriptions of *L. salmonis* have led to the differentiation of *L. uenoi*.

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A NEW SPECIES OF LECITHOPHYLLUM FROM NORTH PACIFIC FISHES WITH A CONSIDERATION OF THE TAXONOMY OF THE GENERA LECITHOPHYLLUM, APONURUS, AND BRACHADENA (TREMATODA: HEMIURIDAE)[1]

L. MARGOLIS

Abstract

Lecithophyllum anteroporum n. sp. is described from *Merluccius productus* from British Columbia waters. Other hosts in the North Pacific are *Oncorhynchus nerka* and *O. gorbuscha*. The status of the genera *Lecithophyllum* Odhner, *Aponurus* Looss, and *Brachadena* Linton, and allocation of species within these genera are discussed. Keys to separate the three genera and the four species of *Lecithophyllum* are presented. *Aponurus intermedius* Manter is transferred to *Lecithophyllum* with *L. fuscum* Yamaguti as a synonym.

Eight specimens of an apparently undescribed species of *Lecithophyllum* were collected from the hake, *Merluccius productus* (Ayres), caught off the mouth of the Fraser River, British Columbia, on October 25, 1957. One and three specimens, respectively, were found in the stomachs of two hake and the other four specimens were recovered from the stomach washings of the remaining two fish, which were examined together. Prior to collecting the trematodes from *M. productus* the same species had been taken from adult *Oncorhynchus nerka* (Walbaum) and *Oncorhynchus gorbuscha* (Walbaum), two species of Pacific salmon.

Data on the occurrence of the trematode in *O. nerka* and *O. gorbuscha* are given in Table I. A total of 31 specimens, 25 from *O. nerka* and 6 from *O. gorbuscha*, were encountered, representing infections in 15 of 1389 *O. nerka*, and 2 of 1017 *O. gorbuscha* examined. The *Oncorhynchus* spp. were taken in 1955 and 1956 in the North Pacific area from the North American coast to the Sea of Okhotsk and Hokkaido. The salmon were frozen immediately after capture and subsequently shipped to the Fisheries Research Board, Biological Station, Nanaimo, where they were thawed and examined. The co-operation of the Japanese Fisheries Agency, the United States Fish and Wildlife Service, and various staff members of this Station in providing the salmon samples, as part of the research program of the International North Pacific Fisheries Commission, is gratefully acknowledged.

The low incidence of *Lecithophyllum* in *Oncorhynchus* spp. suggests that these fishes are incidental hosts. *Merluccius productus* is probably a normal definitive host.

Of the specimens collected from *M. productus*, four were killed and fixed by being dropped into hot (70° C) alcohol – formalin – acetic acid solution and four into hot Bouin's solution. Those from *Oncorhynchus* were killed during the freezing of the host and subsequently were fixed in 10% formalin.

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TABLE I

RECORDS OF COLLECTION OF *Lecithophyllum anteroporum* N. SP. FROM *Oncorhynchus nerka* AND *O. gorbuscha* IN THE NORTH PACIFIC

Locality	Date of capture	No. of fish examined	No. of fish infected	No. of specimens in each fish
Oncorhynchus nerka				
Fraser R., British Columbia	4–8–1955	25	2	1, 10
Rivers Inlet, British Columbia	18–7–1956	25	1	1
Skeena R., British Columbia	21–7–1955	25	2	1, 1
Skeena R., British Columbia	18–7 to 5–8–1956	25	1	1
Seldovia, Cook Inlet, Alaska	21–7–1955	25	1	1
Karluk R., Kodiak Is., Alaska	22–6–1956	25	1	1
51° N. lat., 175° W. long.	8–8–1956	20	1	1
Attu, Aleutian Is., Alaska	4–8–1955	25	1	1
48° 43′ N. lat., 170° 23′ E. long.	18–5–1955	7	1	1
48° 35′ N. lat., 168° 36′ E. long.	1–6–1955	10	1	1
51° 04′ N. lat., 164° 08′ E. long.	25–7–1956	25	1	2
51° 40′ N. lat., 159° 50′ E. long.	5–8–1956	25	1	1
51° 55′ N. lat., 154° 13′ E. long.	6–7–1956	25	1	1
Oncorhynchus gorbuscha				
Fraser R., British Columbia	3–10–1956	16	1	1
Ketchikan, Southeast Alaska	7–8–1956	25	1	5

The trematodes were stained with Gower's alum carmine or Grenacher's borax carmine, cleared in beachwood creosote, and mounted in Canada balsam. One of the specimens from *M. productus* (fixed in Bouin's) and four specimens from *Oncorhynchus* were sectioned sagittally or frontally and stained in Delafield's haematoxylin. Only the Bouin's-fixed specimen gave satisfactory sections.

The following description is based on the specimens from *M. productus* since stained whole mounts, as well as sectional material, were superior to those prepared from specimens collected from *Oncorhynchus*.

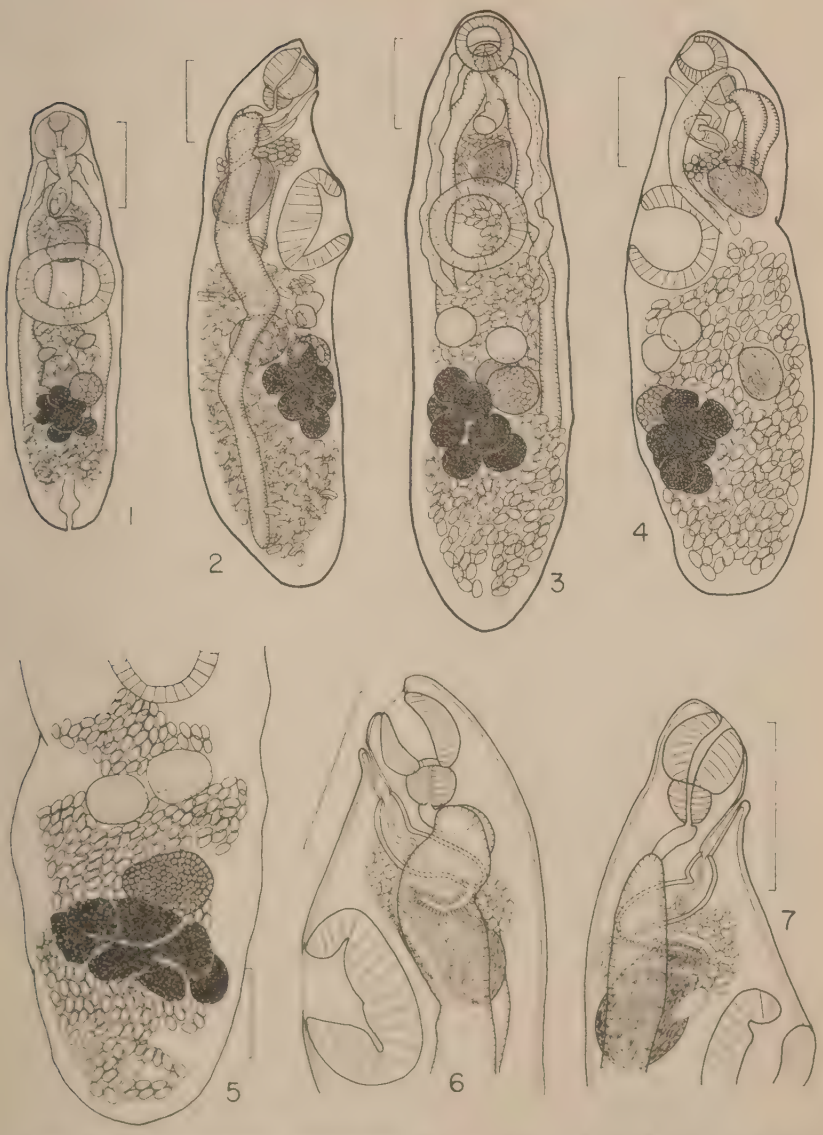
Lecithophyllum anteroporum n. sp.

(Figs. 1–6)

Description

The body is more or less cylindrical with broadly rounded anterior and posterior extremities. In ventral aspect the sides are nearly parallel behind the acetabulum and taper anterior to the acetabulum. Mature specimens measure 0.97 to 1.47 mm in length with a maximum breadth or depth of 0.263 to 0.355 mm. The forebody is about $\frac{1}{4}$ to $\frac{3}{10}$ of the total body length. The length of the subterminal oral sucker is usually close to $\frac{1}{2}$ the length of the acetabulum.

The subglobular pharynx, about $\frac{1}{2}$ of the length of the oral sucker, lies posterodorsal to this sucker. A short esophagus, directed posterodorsally, gives rise to two wide caeca that become narrower posteriorly. They run dorsally and terminate near the posterior extremity of the body, at about the same level or a little in front of the hindmost uterine loops.



FIGS. 1–6. *Lecithophyllum anteroporum* n. sp. FIG. 1. Ventral view of specimen from *Merluccius productus*. FIG. 2. Lateral view of specimen from *M. productus*. FIG. 3. Ventral view of specimen from *Oncorhynchus nerka*. FIG. 4. Lateral view of specimen from *O. nerka*. FIG. 5. Hindbody of specimen from *O. nerka*, compressed before fixation. FIG. 6. Lateral view of forebody of specimen from *M. productus*. FIG. 7. *Lecithophyllum sphaerolecithum* (Manter). Lateral view of forebody. All figures drawn with the aid of a camera lucida. Scale = 0.2 mm.

The diagonally arranged tests are oval to round in outline and lie a little behind the acetabulum near the ventral surface of the body. Although their positions relative to one another are variable, the left testis is anterior and dorsal to the right one more frequently than not. The size of the testes is not related to body size, smaller specimens sometimes having larger testes than larger specimens.

The pear-shaped to ovoidal seminal vesicle lies dorsal and partially anterior to the acetabulum with its long axis directed obliquely dorsoventrally and the broad base extending behind the anterior margin of the acetabulum. From the anteroventral apex of the seminal vesicle a short narrow duct leads to the prostatic duct. The latter follows a dorsal or obliquely dorsal course, sometimes folding back on itself, to enter the base of the hermaphroditic pouch subterminally, where it joins the hermaphroditic duct. The prostatic duct varies in relative length from about $\frac{2}{3}$ to almost equal the length of the hermaphroditic pouch. The prominent prostatic cells are not uniformly distributed along the entire length of the prostatic duct but are arranged mainly in two groups, a dorsal and a ventral. Some of these prostatic cells have long ducts which open into the mid-region of the prostatic duct.

The coiled or folded hermaphroditic duct, enclosed in the hermaphroditic pouch, opens into a prominent genital atrium. Its basal portion is enlarged and receives the pars prostatica and uterus. The portion that protrudes into the genital atrium is of greater diameter than the adjacent portion enclosed in the hermaphroditic pouch. The length of the hermaphroditic duct is difficult to determine because of its folds and coils, but it is at least equal to the combined lengths of the hermaphroditic pouch and the genital atrium, thus exceeding the length of the pars prostatica. The elongate–pyriform hermaphroditic pouch is directed anteroventrally from the end of the prostatic duct and is frequently bent back on itself before reaching the base of the genital atrium. The base of the hermaphroditic pouch is always located considerably more than one-half the distance from the genital pore to the acetabulum.

The genital atrium, about $\frac{1}{3}$ to $\frac{2}{3}$ the length of the hermaphroditic pouch, opens to the exterior, as the genital pore, in a median, transversely oval slit, ventral to the oral sucker.

The ovary, oval to round in outline, is situated behind the testes, a little to the left or right of the mid-line, close to the ventral surface of the body. It is larger than the testes, the difference in size being more pronounced in the larger specimens. The seminal receptacle lies dorsal and partially anterodextral (or anterolaevo) to the ovary and is about equal in size with it except in the smallest and youngest (based on number of eggs in the uterus) specimen in which it is only one-half the diameter of the ovary.

The vitellaria consist of seven lobes, round or oval to pyriform in outline, arranged in two distinct groups of four and three lobes each. The group of four lobes lies to the right or left of the ovary (depending on whether the ovary is to the left or right of the mid-line) and partially posterior to it, and

the other group lies behind these and the ovary. The seven lobes do not unite at a common central point, but in some specimens the lobes within each group were clearly observed to be united. There is variation in the size of individual vitelline lobes in a specimen, but there is no difference between the sizes of lobes in the two groups. All vitelline lobes are smaller than the ovary.

TABLE II

MEASUREMENTS (IN MILLIMETERS, EXCEPT EGGS IN MICRONS) OF MATURE *Lecithophyllum anteroporum* N. SP. FROM *Merluccius productus* AND *Oncorhynchus* SPP.

	M. productus		*Oncorhynchus* spp.	
	Range	Mean*	Range	Mean*
Total length	0.97 –1.47	1.19 (8)	0.91 –1.49	1.23 (23)
Maximum breadth or depth	0.248–0.355	0.293 (8)	0.278–0.477	0.360 (23)
Forebody length	0.291–0.419	0.328 (8)	0.277–0.398	0.334 (23)
Oral sucker				
Length	0.103–0.128	0.111 (8)	0.096–0.148	0.115 (23)
Width or depth	0.101–0.136	0.118 (8)	0.095–0.135	0.116 (23)
Pharynx				
Length	0.047–0.071	0.058 (8)	0.052–0.084	0.065 (23)
Width and depth	0.058–0.085	0.074 (8)	0.051–0.082	0.066 (23)
Esophagus length	0.063–0.077	0.068 (6)	0.054–0.079	0.064 (7)
Acetabulum				
Length	0.182–0.248	0.214 (8)	0.176–0.280	0.218 (23)
Width	0.186–0.254	0.222 (4)	0.176–0.276	0.208 (8)
Depth	0.126–0.189	0.163 (8)	0.155–0.245	0.203 (20)
Ratio oral sucker length to acetabulum length	1:1.67–1:2.10	1:1.92 (8)	1:1.76–1:2.41	1:1.91 (23)
Seminal vesicle				
Length	0.126–0.221	0.167 (8)	0.126–0.245	0.155 (21)
Maximum width	0.087–0.122	0.098 (8)	0.064–0.128	0.095 (23)
Prostatic duct length	0.111–0.150	0.132 (7)	0.089–0.146	0.111 (13)
Hermaphroditic pouch				
Length	0.158–0.186	0.169 (7)	0.114–0.178	0.150 (14)
Maximum width	0.041–0.071	0.057 (8)	0.040–0.073	0.052 (20)
Genital atrium length	0.063–0.122	0.080 (7)	0.082–0.160	0.123 (13)
Testes				
Length	0.030–0.090	0.062 (8)	0.071–0.150	0.100 (20)
Width or depth	0.047–0.095	0.067 (8)	0.076–0.170	0.113 (20)
Ovary				
Length	0.084–0.131	0.104 (8)	0.095–0.176	0.123 (18)
Width or depth	0.079–0.122	0.095 (8)	0.098–0.181	0.131 (18)
Seminal receptacle				
Length	0.085–0.131†	0.107 (7)	0.071–0.142	0.108 (8)
Width or depth	0.088–0.147†	0.109 (7)	0.071–0.119	0.095 (8)
Vitelline lobes				
Length	0.035–0.119	0.071 (8)	0.064–0.126	0.092 (16)
Width or depth	0.038–0.098	0.070 (8)	0.064–0.114	0.090 (16)
Postvitelline body length	0.152–0.369	0.254 (8)	0.184–0.361	0.270 (23)
Ova				
Length	42.7–61.6	49.8 (8)‡	38.0–60.0	45.9 (25)‡
Width	23.7–30.0	25.5 (8)‡	20.5–28.4	25.5 (25)‡
Mean length in individual specimens	44.8–55.8	49.8 (8)‡	40.9–54.6	45.9 (25)‡
Mean width in individual specimens	24.6–25.9	25.5 (8)‡	22.2–27.5	25.5 (25)‡

*Number of specimens upon which the mean was based is shown in parentheses.

†The [illegible] specimen in which the seminal receptacle, 0.044×0.046 mm, was only about one-half the diameter of the ovary.

‡Ten eggs measured in each specimen.

The uterus, filled with ovoidal, operculate ova, occupies most of the available space in the hindbody. In the forebody a single loop of the uterus passes anteroventrally between the acetabulum and the seminal vesicle. It turns dorsally over the seminal vesicle, closely following the pars prostatica, and enters the base of the hermaphroditic pouch to join the hermaphroditic duct ventrolateral to the pars prostatica. The average egg size based on measurements of 10 eggs in each of eight specimens is 49.8μ by 25.5μ. The complete range in size is 42.7 to 61.6μ in length by 23.7 to 30.0μ in width. Although the variation in length is 18.9μ and in width 6.3μ, the maximum variation in any one specimen is 9.4μ in length and 4.7μ in width. The mean egg size of individual specimens varied from 44.8 to 55.8μ in length and 24.6 to 25.9μ in width.

The excretory pore is terminal. The main excretory duct bifurcates in the testicular region, giving rise to two branches which run anteriorly in the lateral fields, ventral to the caeca, and unite dorsal to the pharynx. A bulbous enlargement of the main duct occurs a short distance anterior to the pore.

Measurements of mature specimens of *L. anteroporum* from *M. productus* and *Oncorhynchus* spp. are given in Table II.

Some differences are noted between the specimens from *Merluccius* and those from *Oncorhynchus*. Specimens from the latter host genus are stouter, have a pharynx that is more nearly spherical, a greater ventrodorsal diameter (depth) of the acetabulum, generally larger gonads and vitellaria, and frequently slightly smaller eggs. The vitelline lobes, although usually smaller than the ovary, as in the specimens from *Merluccius*, occasionally are as large as the ovary. These differences between specimens from the two host genera are not important enough to warrant consideration of the existence of two distinct species. The development in different hosts and/or the effects of the different methods of killing and fixation probably account for the observed dissimilarities.

Type host: Merluccius productus (Ayres), hake (family Merlucciidae).

Other hosts: Oncorhynchus nerka (Walbaum), sockeye salmon; *Oncorhynchus gorbuscha* (Walbaum), pink salmon (family Salmonidae).

Type locality: Mouth of the Fraser River, British Columbia.

Geographical distribution: North Pacific, from British Columbia and Alaska to the Sea of Okhotsk.

Type specimens: Holotype and paratypes to be deposited in the National Museum, Ottawa, Canada.

Discussion

The genus *Lecithophyllum* was created by Odhner (21) to accommodate *Distoma botryophoron* Olsson, 1868 (24). Three other species have since been assigned to this genus: *L. fuscum* Yamaguti, 1938 (31), *L. sphaerolecithum* (Manter, 1925) Manter, 1947 (14, 15, 19), and *L. pyriforme* (Linton, 1910) Yamaguti, 1953 (9, 34).

Manter (19) reduced *L. fuscum* to the synonymy of *Aponurus intermedius* Manter, 1934 (17). Although a genital atrium was not described for *A. intermedius*, as in *L. fuscum*, its presence is indicated in the illustration since the genital pore is shown some distance in front of the hermaphroditic pouch. There have been conflicting concepts of the two genera but the presence of a prominent genital atrium in *Lecithophyllum* and its absence in *Aponurus* appears to be the significant difference between them as will be noted later in this paper. Therefore, *Aponurus intermedius* should be transferred to *Lecithophyllum*, with *L. fuscum* as a synonym.

Lecithophyllum sphaerolecithum was returned to its original generic designation, *Aponurus*, by Yamaguti (34) because a genital atrium had not been described. Through the courtesy of Mr. Allen McIntosh of the United States Bureau of Animal Industry, Beltsville, Maryland, I have been able to examine the two type specimens[2] upon which Manter's description was based and have found that a genital atrium does in fact exist (see Fig. 7). Consequently the species is truly a *Lecithophyllum*.

Lecithophyllum pyriforme should be excluded from this genus because of the difference in structure of the vitellaria.

Thus, four species, including the new one described herein, seem to constitute the genus *Lecithophyllum* at the present time.

Key to the Species of *Lecithopyllum*

1. Hermaphroditic pouch globular; prostatic duct four to five times its length..... ..... *L. intermedium* (Manter, 1934), n. comb.
2. Hermaphroditic pouch elongate; prostatic duct shorter than it.....3
3. Seminal vesicle entirely anterior to acetabulum; length of adults 2–3 mm..... ..... *L. botryophorum* (Olsson, 1868) Odhner, 1905
4. Seminal vesicle at least partially dorsal to acetabulum; length of adults less than 1.5 mm. .5
5. Genital pore at level of mid-pharynx; group of three vitellaria anterior to and smaller than group of four..... *L. sphaerolecithum* (Manter, 1925) Manter, 1947
6. Genital pore at level of oral sucker; group of four vitellaria anterior to group of three and of equal sizes..... *L. anteroporum* n. sp.

In re-examining the type specimens of *L. sphaerolecithum* certain errors or omissions in Manter's account of this species, based on the 1.1 mm specimen, were noted. These are corrected in the following statements. Since Manter did not recognize a genital atrium, his description and measurements of the hermaphroditic pouch actually apply to this structure combined with the genital atrium. The hermaphroditic pouch measures 0.142 mm in length by 0.052 mm in maximum breadth and the genital atrium is 0.074 mm in length. Manter gave the length of the prostatic duct as about 0.19 mm but by measurement of the same structure is only 0.108 mm. The prostatic cells are not uniformly distributed about the prostatic duct but are arranged similarly to those in *L. anteroporum*. A short narrow duct joins the seminal vesicle and the prostatic duct. The posterior margin of the acetabulum is 0.53 (not 0.43) mm from the anterior end. The scale accompanying Manter's (15) Fig. 70 bears out this corrected measurement. The seminal vesicle is 0.158

[2] The 1.1 mm specimen is laterally mounted and the larger specimen is transversely sectioned.

mm in length by 0.098 mm in maximum breadth in lateral view. The post-vitelline body length is 0.209 mm. Measurements of 10 eggs selected at random ranged in size from 55.3 to 62.4 μ by 26.8 to 28.4 μ, with a mean of 58.9 μ by 27.5 μ. The oral sucker is 0.095 mm long and the ventral sucker 0.170 mm long, with a ratio of 1:1.78. The gonads and vitellaria are slightly smaller than indicated by Manter. These slightly smaller measurements of gonads, vitellaria, and suckers may be due to the fact that Manter's measurements were probably taken from a temporary ventral mount of the specimen and hence represent measurements along a different axis of these structures. The smaller egg dimensions are probably the result of shrinkage.

With the amended description of *L. sphaerolecithum* it is apparent that it is the closest relative of *L. anteroporum*, differing from this species as noted in the key. In addition, the seminal receptacle in fully mature individuals is about the same size as the ovary in *L. anteroporum* whereas it is only one-half the diameter of the ovary in *L. sphaerolecithum*. However, since the wall of the seminal receptacle is muscular its size may be largely dependent on the amount of sperm stored in it. The mean egg size of *L. anteroporum* appears to be smaller than that of *L. sphaerolecithum*, although there is considerable overlap in the ranges.

The lack of an adequate description of the terminal reproductive ducts in *L. botryophorum* precludes a detailed comparison of this species with *L. anteroporum*. Olsson's (24) illustration from the ventral aspect suggests that the ducts from the genital pore to the base of the seminal vesicle are linearly arranged, in contrast to the sigmoidal arrangement of these structures in *L. anteroporum* as seen in lateral view. However, a lateral view of *L. botryophorum* might be more revealing of the arrangement of the terminal genital ducts. Odhner (21) gave the length of the genital sinus (hermaphroditic duct) as 0.30 mm. Does this measurement actually refer to the hermaphroditic duct or is it the length of the sinus sac (hermaphroditic pouch), as interpreted by Dawes (2)? The hermaphroditic pouch is more readily measurable than the hermaphroditic duct, which lies coiled within the pouch, as in *L. anteroporum*. A true measurement of the length of the duct would be difficult to obtain unless it was completely extended, but its length must be at least equivalent to the combined lengths of the hermaphroditic pouch and the genital atrium. The length of this latter structure has not been cited. The need for clarification of the arrangement and lengths of the terminal genital ducts in *L. botryophorum* is thus apparent.

The Genera *Lecithophyllum*, *Aponurus*, and *Brachadena*

The genera *Aponurus* Looss, 1907 (11, 12) and *Brachadena* Linton, 1910 (9) are closely allied to *Lecithophyllum* Odhner, 1905 (21). Neither Looss nor Linton compared or attempted to differentiate their genera from *Lecithophyllum*. Subsequent attempts at differentiation and allocation of species within these genera have not been universally accepted. Odhner (23, footnote, page 6) noted that *Lecithophyllum* possesses a well-developed genital

atrium which is absent in *Aponurus*. Nevertheless he suggested that *Aponurus sphaerolecithus* Manter may actually belong to *Lecithophyllum* because of the large egg size, although it was not known at that time that a genital atrium is present in this species.

Manter (19) suggested that the two genera be separated on the basis of egg size and length of the hermaphroditic duct. He did not make use of the presence or absence of a genital atrium. *Lecithophyllum* was characterized as possessing a hermaphroditic duct longer than the pars prostatica and eggs 55 to 65 μ long. Species in which the egg size is less than 55 μ *or* in which the hermaphroditic duct is shorter than the pars prostatica were placed in *Aponurus*. Manter's concepts, which were accepted by Chauhan (3) and Skryabin and Gushanskaya (*in* Skryabin (26)), required the transfer of *A. sphaerolecithus* to *Lecithophyllum* and the transfer of *L. fuscum* to *Aponurus*. The discovery of *L. anteroporum* indicates that Manter's suggestions for distinguishing the two genera are impractical. In this species the hermaphroditic duct is longer than the pars prostatica but the egg length (38 to 61.6 μ) includes part of the range of *Lecithophyllum* as well as part of the range (up to 45 μ) of *Aponurus* species as cited by Manter.

Yamaguti (34), reverting in part to Odhner's (23) differentiation of the two genera, distinguished *Lecithophyllum* from *Aponurus* by the presence of a genital atrium and by the character of the hermaphroditic duct. For *Lecithophyllum* he described the duct as constricted into two portions, an enlarged basal portion and a dilated distal portion opening into the genital atrium, whereas in *Aponurus* it was described as tubular, without a constriction. A constriction of the hermaphroditic duct into two regions was not mentioned by Olsson (24) in his original description of *L. botryophorum*, the type species, nor in Odhner's (21) redescription of type material.[3] It therefore cannot be accepted yet as a generic character of *Lecithophyllum* in spite of the presence of this type of hermaphroditic duct in the other three species of the genus.

The essential character separating *Lecithophyllum* from *Aponurus* appears to be the presence of a well-developed genital atrium in the former genus and its absence in the latter one.

Yamaguti (34) relegated *Brachadena* to the synonymy of *Lecithophyllum*. However, the presence or absence of a genital atrium has not been established for *Brachadena*. Furthermore, the vitellaria in the type and only species, *B. pyriformis* Linton, 1910 (1, 9, 10, 13, 16, 19), have been described or illustrated by various authors (under this name or one of its synonyms as listed by Manter, 19) as consisting of seven elongate lobes united centrally. Olsson (24) originally described the vitellaria in *L. botryophorum* as composed of seven pear-shaped lobes united centrally, but, Odhner (22), in re-examining new material of this species, found that the vitellaria actually consist of two groups, one with three lobes and the other with four lobes, the lobes within

[3] Yamaguti (34) cited Linton's (7, 8) records of *L. botryophorum*. Linton (10) and Manter (16, 19) had already re-identified the specimens as belonging to no less than three species: ***Lecithaster gibbosus, Leithaster confusus,*** and ***Brachadena pyriformis.***

each group being united. Looss (12) in his generic definition of *Aponurus* stated that the vitelline follicles are shaped like irregular spheres, frequently forming a group of three and a group of four. Later in the same paper he commented that in compressed fresh specimens sometimes the vitellaria are no longer recognizable as two distinct groups and the vitellaria then resemble those of *Lecithaster* since all lobes *seem* to originate at one point. He further wrote that (free translation) "I believe that this is indeed the case, even if the direct proof on mounted and cleared individuals cannot be produced because of the unfavourable position of the follicles." The use of the word 'seem' indicates uncertainty on Looss's part in interpreting the actual condition of the vitellaria in compressed specimens and he has presented no conclusive evidence that the vitelline lobes in *A. laguncula* are centrally united. Since the globular or subglobular vitellaria of *Aponurus* and *Lecithophyllum* are distributed in both depth and width of a cylindrical body, flattening of a specimen, particularly before fixation, considerably alters their shape and arrangement, the extent of this alteration depending on the amount of flattening. The vitelline lobes in a compressed specimen of *L. anteroporum* from *O. nerka*, although changed in shape and arrangement, do not give the impression of originating from a central point (Fig. 5). This was also borne out by study of temporary mounts of individual specimens, viewed ventrally, dorsally, laterally, or from various ventrolateral or dorsolateral aspects. Manter (15) also stated that he could find no evidence from serial transverse sections that the vitelline lobes of *L. sphaerolecithum* are centrally united. From the descriptions of the various species of *Aponurus* and *Lecithophyllum* it must be concluded that the vitellaria in these two genera consist of seven spherical to subspherical or short pyriform lobes which are not centrally united but which are frequently arranged in two groups, one of four lobes and the other of three lobes.[4] The members within each group, at least in *Lecithophyllum*, may be united.

On the basis of vitelline structure it is advisable, for the present, to retain *Brachadena* as an independent genus. Bravo-Hollis (1), in refuting Yamaguti's decision to unite *Brachadena* with *Lecithopyhllum*, tabulated several differences between them which seem to be only of a specific nature. She did not compare the structure of the vitellaria.

A provisional key to separate the three genera may be given as follows:

1. Vitellaria consisting of seven elongate lobes united centrally.....*Brachadena*
2. Vitellaria consisting of seven spherical, subspherical, or short pyriform lobes, not centrally united, but frequently arranged in two groups of three and four:
   a. Well developed genital atrium present.....*Lecithophyllum*
   b. Genital atrium lacking.....*Aponurus*

[4]*Aponurus vitellograndis* Layman has been described as having star-shaped vitellaria. The lobes, as illustrated by Layman (5), are pyriform, but much shorter than in *B. pyriformis*. This species may yet find its true position in *Brachadena* but the gross appearance of the vitellaria is considerably different from that in *B. pyriformis*. A reinvestigation of the vitellaria in *A. vitellograndis* should be undertaken before a decision is made. It may be discovered that the vitellaria are actually disposed in two groups. Skryabin and Gushanskaya (*in* Skryabin (26)) transferred Layman's species to *Hysterolecitha*, but one of the prominent characters of this genus is a tubular, winding, or coiled seminal vesicle which excludes *A. vitellograndis* from it.

As already noted, *Brachadena* is a monotypic genus and *Lecithophyllum* contains four species. The genus *Aponurus*, apparently the commonest of the three genera, has had 16 species assigned to it, of which one, *A. bowersi* Leiper and Atkinson, 1914 (6), does not belong to this genus because the vitellaria consist of two compact masses[5] and two others, *A. intermedius* and *A. sphaerolecithus*, have been transferred to *Lecithophyllum*. The remaining 13 species are *A. laguncula* Looss, 1907 (11, 12, 19, 33), *A. tschugunovi* Isaichikov, 1927 (4, 29), *A. vitellograndis* Layman, 1930 (5), *A. rhinoplagusiae* Yamaguti, 1934 (30), *A. brevicaudatus* Yamaguti, 1934 (30), *A. callionymi* Yamaguti, 1938 (31), *A. acropomatis* Yamaguti, 1938 (31), *A. breviformis* Srivastava, 1939 (28), *A. bengalensis* Srivastava, 1939 (28), *A. trachinoti* Manter, 1940 (18, 20), *A. argentini* Polyanskii, 1952 (25), *A. carangis* Yamaguti, 1952 (32), and *A. synagris* Yamaguti, 1953 (33).

The possibility of *A. vitellograndis* being a member of another genus has been noted earlier in this paper. The terminal reproductive ducts have been inadequately described and illustrated in *A. tschugunovi* so that its position within the genus cannot be verified. The description of the terminal genital ducts in *A. argentini* is not in accordance with the concept of *Aponurus*. Polyanskii (25) did not mention a hermaphroditic duct and pouch but rather described a cirrus and the uterus extending independently to the genital pore. Other characters are in agreement with the concept of *Aponurus* or *Lecithophyllum* and the description of the terminal genital ducts may be in error. In fact this species, which was taken from the same host (*Argentina silus*) and general locality as *L. botryophorum*, is identical with the latter in all respects except for the terminal genital ducts, and subsequent studies may prove the two species to be synonymous. All other species listed above have vitellaria consisting of seven spherical to short pyriform lobes, not centrally united, but commonly in groups of four and three, and a hermaphroditic duct enclosed in a pouch that starts immediately behind the genital pore.

In future morphological studies on members of the *Lecithophyllum–Aponurus–Brachadena* complex, particular attention should be focussed on the structure of the vitellaria and the terminal genital ducts in order to assist in clarification of the taxonomy of these genera.

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[5]Skryabin and Gushanskaya (*in* Skryabin (27)) transferred *Aponurus bowersi* to *Genolinea*. Since the seminal vesicle and details of the terminal genital ducts have not been described for this species, the transfer to *Genolinea* is unjustified. At the present time the correct generic allocation of *A. bowersi* must remain questionable.

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STUDIES ON MYTILUS EDULIS L. OF THE "CALANUS" EXPEDITIONS TO HUDSON BAY AND UNGAVA BAY

"CALANUS" SERIES No. 16[1]

I. LUBINSKY

Abstract

The "Calanus" expeditions of the Fisheries Research Board of Canada give the first detailed data on the distribution of *Mytilus edulis* L. in Ungava Bay, Hudson Strait, and the northern part of the Hudson Bay. Types of shells of this mollusc and their growth in the above regions are described. The comparison of the growth of *M. edulis* from Canadian Eastern Arctic, west Greenland, and the northwestern shores of the Atlantic Ocean shows that northward from the Canadian Maritime Provinces the growth of *M. edulis* slows down. In the region investigated by "Calanus", growth diminishes from Ungava Bay to Hudson Bay. The dependence of the distribution of *M. edulis* on the types of water masses and its relation to the northern limits of the subarctic zone in the Canadian Eastern Arctic is discussed.

Our knowledge of the distribution of *Mytilus edulis* L.—the edible mussel—in the Canadian Eastern Arctic has hitherto been very limited. This species has previously been recorded from only two localities in Ungava Bay, namely the mouth of Koksoak River (13) and Cape Hopes Advance (59). It has also been reported from an unspecified locality in Hudson Bay (66), from Fullerton, Roes Wellcome (15), Southampton Island (4), and Chesterfield Inlet (39). It was found in Ashe Inlet in the Upper Savage Island in Hudson Strait (66).

The "Calanus" expeditions of the Fisheries Research Board of Canada, 1947–1953, have shown that this mollusc is widely distributed in the region from Port Burwell on the northeastern shore of Ungava Bay to Churchill on the western shore of Hudson Bay. Specimens of *M. edulis* were taken in Ungava Bay, stations 212, 208, 8, 5, 31, 107, 224, 66, 67, and 215; in Wakeham Bay at station 217; in Hudson Bay at stations 525, 528, 504, 555, 513, and 519. The description of the above stations and their location is given by Dunbar and Grainger (22), Grainger (31), and Grainger and Dunbar (32).

Mytilus was collected exclusively in the intertidal zone. Samples contained 5–15 specimens, with the exception of those from Burwell, where 38 specimens were collected. Numerous empty shells of this mollusc were often found scattered on the shores. Sometimes it was difficult to decide whether they originated from contemporary nearby beds or were washed out from raised ancient beaches, which contain numerous shells of this mollusc (39, 49, 52, 63). It is hard to say whether at present *Mytilus* is as abundant as it has been previously. The "Calanus" expeditions have shown it to be abundant only in a few localities: near Port Burwell, along southwestern shores of Ungava Bay, and in Harbour of Nuvuk.

This paper is based on a limited material and has to be regarded as a preliminary report.

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Based on material collected by the "Calanus" expeditions of the Fisheries Research Board of Canada.

TABLE I

DIMENSIONS (IN MM) AND AGE OF SHELLS OF *Mytilus edulis* L. IN HUDSON BAY AND UNGAVA BAY

	Age	L	H	W	H/L	W/L	W/H
				Hudson Bay			
Port Churchill	14	47	25	10	0.53	0.44	0.76
Station 555 L^1	9	37	18	13	0.48	0.35	0.72
	11	40	18	13	0.45	0.32	0.72
	9	38	18	14	0.47	0.37	0.78
	4	27	14	12	0.51	0.37	0.71
	2	21	11	7	0.50	0.33	0.70
Nuvuk Harbour	12	53	29	25	0.54	0.47	0.89
Station 528 L^6	11	48	25	21	0.52	0.43	0.89
Station 525 L^5	8	43	22	20	0.51	0.44	0.98
	10	59	81	25	0.52	0.42	0.80
	10	51	24	20	0.47	0.39	0.85
	5	33	17	15	0.51	0.45	0.88
	0	49	25	22	0.51	0.45	0.88
	4	32	19	14	0.50	0.43	0.79
	4	31	18	15	0.58	0.48	0.83
	3	20	11	10	0.54	0.41	0.90
	3	21	12	9	0.57	0.42	0.75
Wakeham Bay	11	68	35	27	0.57	0.39	0.77
Station 217 L^{11}	10	62	29	27	0.46	0.39	0.93
	10	60	25	26	0.41	0.43	1.04
	7	56	27	24	0.47	0.44	0.93
	8	55	31	23	0.56	0.43	0.76
	7	55	29	22	0.53	0.40	0.76
	7	50	24	23	0.48	0.46	0.95
	8	51	26	25	0.50	0.49	0.56
	5	49	24	21	0.50	0.74	0.80
				Ungava Bay			
Southern shores	11	81	38	36	0.47	0.44	0.94
Station 8 L^{11}	8	76	37	32	0.49	0.42	0.86
Station 31 L^{14}	10	74	34	31	0.46	0.40	0.31
Station 5 L^8	8	64	31	30	0.49	0.46	0.36
	7	67	33	28	0.49	0.41	0.96
Port Burwell	12	72	36	29	0.50	0.40	0.80
Station 67 L^4	10	59	32	24	0.54	0.40	0.79
Station 224 L^{15}	10	62	31	24	0.50	0.40	0.80
Station 66 L^3	11	65	39	25	0.60	0.40	0.76
	8	57	31	22	0.54	0.40	0.70
	9	56	30	23	0.53	0.43	0.78
	8	55	26	21	0.50	0.40	0.78
	4	45	23	19	0.51	0.41	0.83
	6	43	22	20	0.51	0.46	0.90
	5	38	19	18	0.50	0.77	0.50
	4	32	16	14	0.50	0.43	0.87
Cape Hopes Advance	7	50	27	22	0.54	0.44	0.82
Station 212 L^8	7	36	18	18	0.50	0.50	1.07
Station 208 L^7	3	29	15	12	0.51	0.71	0.80
	5	33	15	14	0.57	0.50	0.74
	6	48	23	23	0.49	0.49	1.00
	7	32	18	13	0.56	0.40	0.89
	3	29	16	14	0.55	0.48	0.88
	5	23	18	11	0.56	0.48	0.84
	6	55	28	22	0.50	0.40	0.79
	5	49	24	18	0.50	0.39	0.73
	4	44	27	22	0.57	0.47	0.81
	5	46	26	20	0.56	0.43	0.80
	4	42	18	17	0.55	0.40	0.80

Shell Types of *Mytilus edulis*

Mytilus in our material varies in size, shape, and color from station to station and to some degree within the same station (Table I). Despite this they form three natural groups: *Mytilus* from the southwestern shores of Ungava Bay, those from Hudson Strait, and those from Hudson Bay. The difference in the length of shells in these groups is considerable (Fig. 1). The individuals of the first group are the largest and those of the third group the smallest. The second group is intermediate and the shells comprising this group vary in size but slightly. *Mytilus* from Burwell can be regarded as representative of this group.

Table II summarizes the data on the length of 5-year-old specimens of these groups. The relations of differences of means to the corresponding errors show that the differences in length in these three groups are significant. Specimens of above groups differ also in the shape of their shells. This shape can be characterized by the ratio of height to length (H/L), width to length (W/L), and width to height (W/H). The mean values of indices of shells of the three groups are shown in Table III. The shells of the first group are low and wide (W/H = 0.89), those of the third group narrow and high.

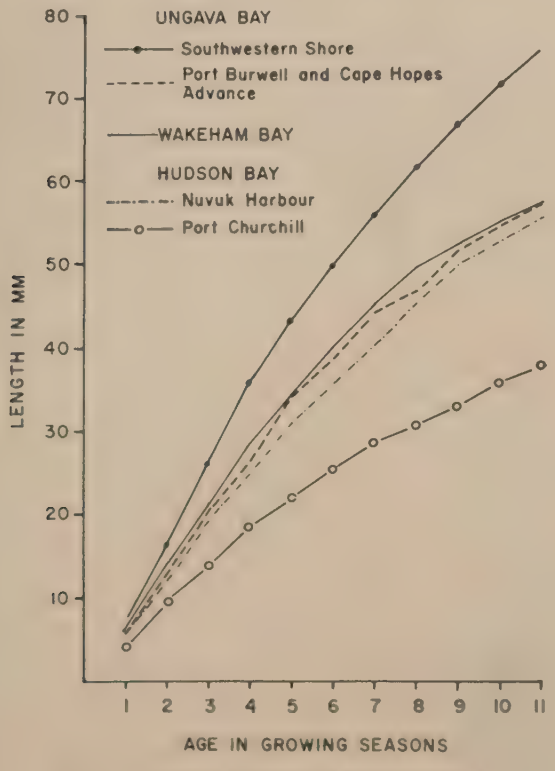


FIG. 1. Growth in length of *Mytilus edulis* L.

The difference of mean values of indices W/H between the second and the third group is insignificant, probably because of the small number of shells from the latter locality. But the difference of mean values of this index of the first group (0.89 ± 0.02; $\sigma = \pm 0.05$) and of the third group (0.75 ± 0.02; $\sigma = \pm 0.05$) is probably significant: $(M_1 - M_2)/\sqrt{\sigma_{M_1}^2 + \sigma_{M_2}^2} = 3.5$. To be significant, for $n = 11$, this relation has to be $\geqslant 3 + 6/(n - 4)$, thus $\geqslant 3.86$.

TABLE II

MEAN LENGTH (IN MM) OF 5-YEAR *Mytilus* FROM DIFFERENT LOCALITIES

Groups	Number of shells	$M \pm \sigma_M$	σ	$(M_1 - M_2)/\sqrt{\sigma_{M_1}^2 + \sigma_{M_2}^2}$
1. Southwestern shores of Ungava Bay	5	41.6 ± 1.23	± 2.70	1–2 gr: 6.1($\geqslant 3.26$)
2. Burwell	22	32.9 ± 0.72	± 3.03	2–3 gr:12.9($\geqslant 3.26$)
3. Churchill	5	22.2 ± 0.53	± 1.18	1–3 gr:21.8($\geqslant 4.0$)

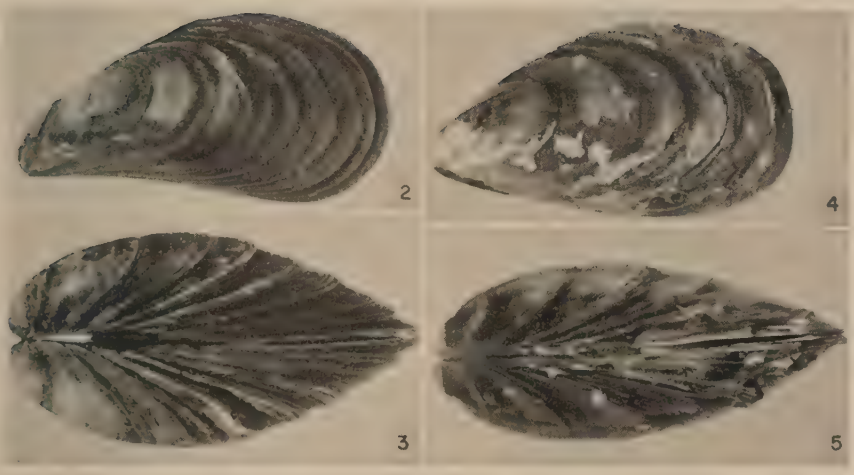
TABLE III

MEAN VALUES OF INDICES H/L, W/L, W/H, OF *M. edulis* FROM DIFFERENT LOCALITIES

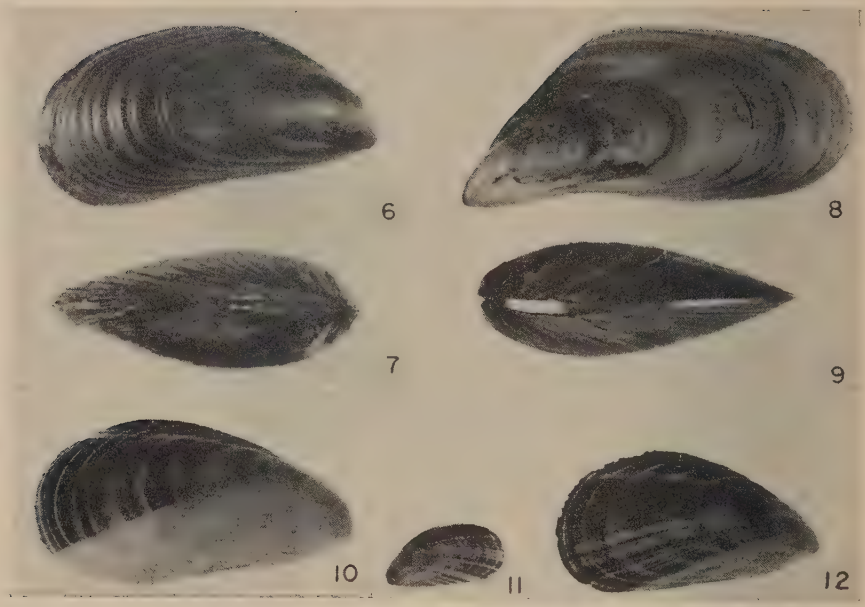
Groups	H/L	W/L	W/H	Number of shells
	Mean values of indices			
1. Southwestern Ungava	0.48	0.43	0.89	5
2. Wakeham Bay	0.49	0.43	0.88	9
Cape Hopes Advance	0.53	0.44	0.83	14
Burwell	0.52	0.43	0.83	22
Nuvuk	0.52	0.44	0.85	11
3. Churchill	0.49	0.37	0.75	6

Beside size and proportions, *Mytilus* of these groups differ also in their habitus. Shells from Ungava Bay are heavy, of blue-brown color, their outer surface rough and often damaged. They are flattened dorsoventrally, with very broad base. The anterodorsal ridge and the upper angle are smooth (Figs. 2, 3). The anterior part of the shell is often bent downward. The largest specimen is 89 mm long and 11 years old. The shells of the second group are also heavy, the periostracum dark brown to light gray brown, often lustrous. The marks of arrested growth are clearly visible as dark lines (Figs. 4, 5). The shape of shells is variable. The largest specimen is 72 mm long and 12 years old. *Mytilus* from Churchill are small, black, lustrous. The marks of arrested growth are visible as step-like sculpture on the surface of the shell (Figs. 6, 7). The shells are narrow and their base in transverse section is V-shaped.

PLATE I



FIGS. 2–5 *Mytilus edulis* L. Figs. 2, 3. From southern shores of Ungava Bay, length 8 cm. Figs. 4, 5. From Burwell, length 7 cm.



FIGS. 6–9. *Mytilus edulis* L. Figs. 6, 7. From Churchill, Hudson Bay, length 4.2 cm. Figs. 8, 9. From Liverpool Bay, mouth of Mackenzie River, length 7 cm. Figs. 10–12. *Mytilus pellucidus* Penn. Fig. 10. From Mansel Island, Hudson Bay, length 6 cm. Fig. 11. From Churchill, length 2 cm. Fig. 12. From Diana Bay, Hudson Strait, length 5 cm.

The peculiarity of the shells of the first and partially of the second group is their diverging beaks. These protrude as two tapering tubercles and may reach a length of 3 mm with a distance between the apices of up to 4 mm. The divergence of beaks is more pronounced in dorsoventrally flattened shells with broad base.

Mytilus with diverging beaks were described over a century ago by Lamarck (38(7), p. 46) as *Mytilus borealis*. This *Mytilus* "habite l'ocean boréale de l'Amérique, côté de New York. Aspect de la moule commune ou commestible, mais beaucoup plus grande. Elle en diffère par ses crochets . . . (diverging beaks-Auct.)." Lamarck's specimen was larger than *Mytilus* from Ungava Bay. Similar shells were later described by Reeve (51) in the legend to the "species 20", depicted in his Plate 6: "*Mytilus borealis*—the northern *Mytilus*. A light ovale, swollen shell of a dark shining olive-black colour, tinged with violet at the umbones, which are somewhat removed from each other. Hab. Newfoundland". Shells from Ungava Bay are in many respects similar to those described by Lamarck and Reeve.

According to these authors this peculiar form of *Mytilus* is a separate species. However, in our material it is connected by intermediate forms to the usual form with beaks in close contact. Its peculiar shape can be interpreted as an adaptation to habitats exposed to the mechanical action of ice and currents. Flattening of the shell lessens its lateral surfaces, whereas the widening of its base secures a better attachment. The beaks diverge as a result of extreme widening and bending of the shell. This form is found in the Ungava Bay, where the tidal currents are very strong. The high environmental plasticity of *Mytilus* is well known. It is interesting that some forms of *M. galloprovincialis* Lam. from the Gulf of Naples (2) are similar to those in Ungava Bay.

The second form of *Mytilus* in the "Calanus" collections is *M. pellucidus* Pennant, 1777, with a beautiful pattern of dark purple rays on the shell (Figs. 10, 11, 12). The rays are clearly visible in thinner shells and in those deprived of periostracum. In some of them the pattern expands over the whole surface but usually is represented by a single narrow ray, visible in transmitted light.

Dodge (17) points out some characters peculiar to *M. pellucidus*, which are so constant that he is "strongly inclined to consider it a good species." The presence of rays, however, in shells from our material is not correlated with morphological characters mentioned by Dodge; for example in our shells the upper angle is smooth and situated far from the beaks. The shells are usually dark colored; the light-colored shells from Wakeham Bay and Port Burwell do not exhibit rays. The scarcity of material does not allow us to make conclusions on the range of variability of *Mytilus* in Hudson Bay and in Ungava Bay. Further research is needed to understand the reactions of this species to the Arctic environments and to "find the correlation between the morphological differences and conditions of habitat" (57).

Growth of *Mytilus edulis*

Observations on the growth of this mollusc in Hudson Bay and in Ungava Bay are based on 67 specimens. The length of shells in successive growing seasons was measured on the basis of lines of arrested winter growth. The method used was that discussed by Mossop (44, pp. 3, 4). The results of measurements are represented in Fig. 1, where the shell length is plotted against the time in growing seasons. The curves of growth are S-shaped (Figs. 1, 13). The differences in the rate of growth in different localities are obvious from the shape of the curves and from their relative positions. *Mytilus* from southern shores of Ungava Bay show the highest growth rate; those from Churchill, the lowest. In other localities growth rates vary but slightly and fall between these two extremes (Fig. 1). The difference in size between the fast-and slow-growing shells (from southwestern Ungava and from Churchill) is about 4 mm at the end of the first growing season; it increases to 23 mm by the 6th season, and is about 35 mm in the 11th growing season.

The relative sizes of the shells in different localities remains, however, almost constant throughout the life of the mollusc. The length of shells from Churchill is about half that of shells from the western Ungava Bay in the 1st as well as in the 11th growing season. The length of shells from Burwell is 0.79 that of shells from the southwestern Ungava in the first growing season, and 0.85 in the 11th season. As a result of differences in the growth rates,

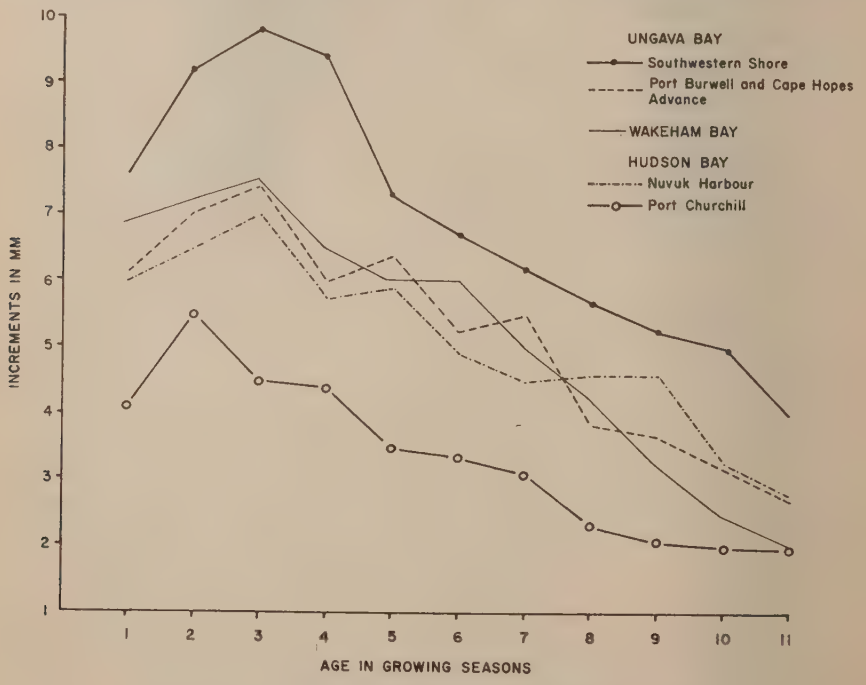


FIG. 13. Annual increments in length of *Mytilus edulis* L.

Mytilus from the southwestern shores of Ungava attain the length of 35 mm in the 4th, from Burwell and Nuvuk Harbour in the 6th, and Churchill in the 10th growing season.

Annual increments as a function of age are represented in Fig 13. The pattern of growth of shells from different localities is almost identical: after a rapid increase in the course of the first three growing seasons with the maximum in the third season, the growth rate gradually decreases. In fast-growing shells the period of rapid growth is prolonged, whereas in slow-growing shells it is limited to the first two growing seasons.

The average annual rate of growth is high in the first two to three growing seasons and decreases with age. In the second five years of life of *Mytilus* it is approximately half of that of the first five years (Tables IV and V).

TABLE IV

THE AVERAGE ANNUAL RATE OF GROWTH OF *M. edulis*

	Age		
	3	5	10
Districts	Average annual rate of growth (in mm)		
Southwestern shores of Ungava Bay	8.8	8.6	7.2
Port Burwell	6.8	6.3	5.5
Churchill	4.6	4.4	3.6

TABLE V

THE AVERAGE ANNUAL INCREMENT DURING THE FIRST AND SECOND 5-YEAR PERIOD OF THE LIFE OF *M. edulis*

	Average annual increment (in mm)	
Districts	First 5 years	Second 5 years
Southwestern Ungava Bay	8.6	4.7
Port Burwell	6.6	3.3
Churchill	4.4	2.4

Discussion

Data on growth of *M. edulis* in the western Atlantic are scarce, and confined mostly to observations on the increment in young mussels (43, 12, 45, 63). In Woods Hole, Mass. (25,54), this mollusc grows to about 75 mm in 5–6 years, in Nova Scotia to the length of 60–80 mm in 4–6 years (43). The shells from Ungava Bay attain this size in about 8–10 years, and in some localities they seldom if ever attain such dimensions (Fig. 1).

The first data on the size of *Mytilus* in consecutive growing seasons were published by Mossop (44). In the first growing season the shells attained the

length of 16 mm in St. Andrews, N.B., and 25 mm in Digby, N.S. Thus they were larger than any shells in our material, which at that age averaged 7–10 mm. The average annual increment of shells in Nova Scotia and New Brunswick varied from 10.8 mm to 16.8 mm, thus was higher than in shells of same age from Ungava Bay (8.6 mm). There is only one note in the literature, that of Mossop (44), on the growth of *Mytilus* in Hudson Bay. According to her the average annual increment of four shells from James Bay, which "appear to be dead when collected", was 8.4 mm, close to that of shells from Ungava Bay.

The distribution and growth of *M. edulis* in the Canadian Arctic is little known. We had the opportunity to examine six specimens of this species collected in the Liverpool Bay near the mouth of the Mackenzie River, now in the collection of the Fisheries Research Board of Canada, Arctic Unit (Table VI). These specimens resemble "species 33*a*" depicted by Reeve (51). The shells are elongated and narrow, with lustrous yellowish-brown periostracum. Valves light, translucent. When deprived of periostracum they are of light blue color with no traces of any pattern. The anterior part of the shell is long, narrow, beaks in close contact. Ridge and angle on the upper margin of the shell are well expressed (Figs. 8, 9). Their growth is fast (Fig. 14). The period of rapid growth extends into the fourth growing season and therefore, from this period on, they are larger than shells of the same age from Ungava Bay. The average annual increment in 6-year-old *Mytilus* from Liverpool Bay is 9 mm, the highest observed in northern latitudes.

TABLE VI

Dimensions (in mm) of *M. edulis* from Liverpool Bay

L	H	W	H/L	W/L
69.2	28.8	23.5	0.41	0.35
59.0	27.0	19.0	0.46	0.32
58.2	28.5	20.1	0.49	0.34
54.2	25.0	17.5	0.46	0.32
54.2	24.6	21.0	0.45	0.37
53.0	24.0	19.0	0.45	0.36
53.5	25.7	18.8	0.48	0.35
37.8	18.5	13.0	0.49	0.34

On the shores of western Greenland *Mytilus* attains considerable size: the largest specimen collected in Godhavn was 78 mm long; in Upernavik, 72 mm (37). In the Thule district the largest specimen measured 93 mm and was probably over 15 years old (62). Other specimens from the same district were: 4 years old and 44 mm long; 5 years old and 29 mm long. The length of 7-year-old specimens varied from 38 to 59 mm. The annual rate of growth calculated on the basis of these data was 5.4–5.8 mm. and attained a maximum of 8.5 mm. It was thus comparable with the rate of growth of *Mytilus* from Ungava Bay and Hudson Bay.

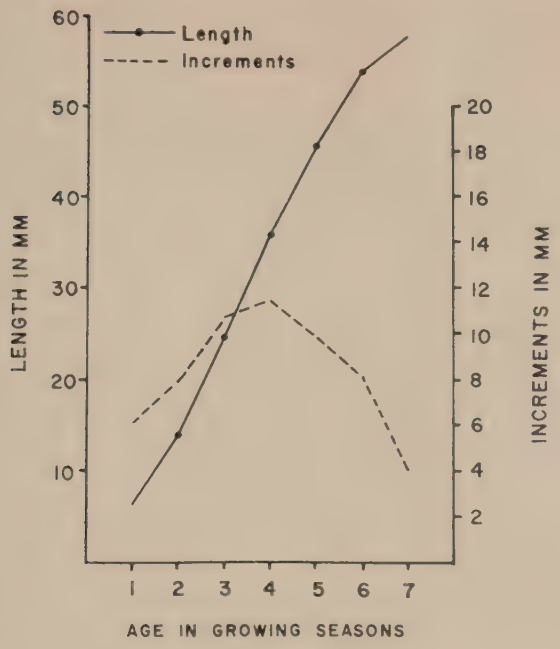


FIG. 14. Growth in length of *Mytilus edulis* L. from Liverpool Bay.

The above data show that north of the Canadian Maritime Provinces the growth of *M. edulis* slows down. This reduction of growth rate in colder waters is a general phenomenon in pelecypods. Thorson (61) pointed out that the growth of arctic species of lamellibranchs is exceedingly slow. The growth of *Mya arenaria* (1, 45, 61), *Mytilus californianus* (10), *M. diegensis*, *Tivela stultorum* (9), *Siliqua patula* (64, 65), as well as of fresh-water bivalves (7), depends on the temperature and on the heat budget of their habitats. It is obvious, moreover, that factors other than temperature influence their growth, e.g. abundance of food, timing of sexual maturity, etc. (48, 61, 8, 9, and many others). Recent observations on the influence of temperature on the activity of poikilotherms show that many species may be relatively independent of this factor. The observations of this type on pelecypods were reported by Spärk (58), Thorson (61), Orton (48), Bruce (5), Fox (26, 28), Rao (50), and their data summarized by Bullock (6). The growth rate, however, does depend on the temperature. "Thus at present there is an apparent contradiction between activity and growth rates" (16, p. 118). As regards gastropods, it has been shown that *Thais emarginata*, *Lacuna carinata*, and *Crepidula nummaria*, while in the egg capsules, grow more rapidly in Alaska than in California (16). Some Cypraeidae and Patellidae are larger in higher latitudes. Other gastropods are larger near the center of their area of distribution, and decrease in size toward the periphery, e.g.

Buccinum undatum, which is often over 100 mm in length near Ireland but attains only half that size in the Barents Sea, near Iceland, and in west Greenland (56).

Weymouth, McMillan and Rich (65) and Weymouth (64) have shown that the northern clams grow more slowly, but live longer and become larger, than the southern ones. The life span of *M. edulis* is not known. The maximum age of specimens in the "Calanus" collections was 14 years; half of the specimens were 10 or more years old. The largest specimen was 8.2 mm long and 11 years old.

Many factors restrict the distribution and inhibit the growth of *Mytilus* in the Arctic: the period of plant and animal growth is short and depends on variable snow and ice conditions (23). Salinity varies considerably and its decrease often affects the growth of *Mytilus*. The inhibition of growth of this mollusc in brackish waters was observed in the Gulf of Bothnia (42), in Kaiser Wilhelm Kanal (3), and in the Baltic Sea (56). In the southern Hudson Bay where the surface salinities are less than $23^0/_{00}$ and mixing of water is poor, *Mytilus* remains small, despite comparatively high temperature. Shells of larger size are found at the mouth of Hudson Bay where salinity attains $32^0/_{00}$, and there is an influx of waters from Foxe Channel and Hudson Strait. In the Ungava Bay, high tides, sweeping across the bay, maintain the circulation, salinity is high and there is an admixture of Atlantic waters (19).

The northern boundaries of the distribution of *M. edulis* in the Eastern Canadian Arctic and in Greenland coincide in general with those of the subarctic zone, as delimited by Madsen (40, 41), and by Dunbar (18, 20, 21). *M. edulis* occurs as far north as Angmagssalik in east Greenland, and Thule in west Greenland. In the Eastern Canadian Arctic it was found, however, near Ponds Inlet (39, 24), thus farther north than the Padloping Island—the northern limit of the subarctic zone in this region (18, Fig. 1). In Hudson Strait it was taken off Southamption Island, and, on the northern shores of Canadian mainland, in the Coronation Gulf. In these last localities the exact position of the boundary of the subarctic zone is not yet known.

Discussing the distribution and growth of *M. edulis* it is necessary to take into consideration the character of water masses in the area. Waters of Canadian Arctic are predominantly of polar character with low salinity and of low temperature. The waters of Hudson Strait, of Ungava Bay, and of west Greenland are of mixed type. The penetration of Atlantic waters can be traced into Ungava Bay, Hudson Strait, and along the shores of west Greenland as far as Thule district (18, 19, 20, 21). As can be inferred from the distribution and growth of *Mytilus*, the best conditions for this mollusc are in the Atlantic waters and to some extent in waters of mixed type. This may explain its better growth in Ungava Bay as compared with Hudson Bay, as well as the penetration of *Mytilus* far northwards along the shores of west Greenland. Its intensive growth in Liverpool Bay may depend on the penetration of Pacific waters, and possibly on the proximity of the estuary of Mackenzie River.

The hydrographic conditions of the Canadian Arctic are complicated, and the latitudinal retardation of the growth of *Mytilus* may be obscured by the peculiarities of its various habitats.

Acknowledgments

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[Contribution from the Fisheries Research Board of Canada, Chemistry Section of the Technological Station at Vancouver]

Marine Sterols. V. Isolation of 7,24(28)-Ergostadien-3β-ol from Starfish[1]

By U. H. M. Fagerlund and D. R. Idler

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A new sterol, 7,24(28)-ergostadien-3β-ol, has been isolated by a chromatographic separation of the azoyl esters prepared from the sterols of the starfish *Pisaster ochraceus*. Starfish sterols contained 19% of this sterol.

The sterols of starfish are generally conceded to be entirely of the C_7-unsaturated type and 7-cholestenol,[2-4] previously found in mammalian skin,[5] recently has been isolated. Hitodesterol has been shown to be identical with α-spinasterol (7,22-stigmastadienol).[6-8] 7-Stigmastenol, a recognized plant sterol,[9] has also been found in starfish.[10-11] Indeed the sterols of starfish have probably been more thoroughly investigated than have those of any other marine invertebrate. A review is scheduled to appear on the sterols of marine invertebrates, including those of starfish.[12]

Previous reports in the present series have described the isolation, characterization and partial synthesis of 24-methylenecholesterol and 24-dehydrocholesterol.[13-16] The former sterol has been suggested to lie on a biochemical pathway common

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FRB No. 546

to sterols of the cholesterol (C_{27}) and ergosterol (C_{28}) type. The structure of the latter sterol suggests that the side-chain double bond of lanosterol has remained unchanged while the ring double bond has migrated from the 8(9) to the 5-position. The structural similarity of the new starfish sterol to 24-methylenecholesterol is apparent. If sterols with a C_7-double bond represent a transition between lanosterol and cholesterol, then the present sterol is of considerable interest because it not only has the C_7-double bond but also the 24-methylene group. The recently reported isolation of 4α-methyl-7,24(28)-stigmastadien-3β-ol from grapefruit[17] suggests a link between the triterpenes and fucosterol in the C_{29}-series. By analogy the new starfish sterol would represent the next intermediate in the transition from triterpenes to 24-methylenecholesterol in the C_{28}-series. Further evidence that the C_7-double bond can be introduced prior to the removal of the 4-methyl group is afforded by the isolation of 4α-methyl-7-cholesten-3β-ol from rat feces[18] and a cactus.[19]

The sterols of marine invertebrates are of particular interest because they offer an isolation–characterization approach to the problem of sterol biogenesis and metabolism. The studies of these organisms are clearly showing that at least in most animals the evolutionary trend is toward cholesterol as the major or sole sterol component of most tissues and organs of the organism. These lower forms of animal life provide a means of studying sterols which thus far have been difficult or impossible to obtain in detectable amounts from more advanced forms of life such as the more common experimental animals.

These studies are also directed toward the attainment of a more solid foundation for the comparative biochemistry of marine invertebrates. The results of the investigations on marine sterols have been previously discussed in relation to the evolutionary development of mollusca and crustacea.[14,16]

The sterol reported in this paper was isolated by chromatography of the steryl azoates (*p*-phenylazobenzoyl esters), prepared from the sterols

(14) D. R. Idler and U. H. M. Fagerlund, *ibid.*, **79**, 1988 (1957).

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(17) F. Sondheimer, Y. Mazur and Anna Weizmann, Abstract of Papers, 133rd Meeting, American Chemical Society.

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of the starfish *Pisaster ochraceus*, on silicic acid–Celite. The isolation of formaldehyde in good yield, following ozonolysis of the sterol, established the presence of a terminal methylene group and this was confirmed by the presence of a strong band at 890 cm.$^{-1}$ and a band of medium strength at 1640 cm.$^{-1}$ in the infrared spectra. The sterol and certain of its derivatives have molecular rotations (Table I) consistent with those of a C_7-double bond.[20] The terminal methylene grouping was established to be in the 24(28)-position employing acid rearrangement followed by ozonolysis (Fig.1)

TABLE I

PROPERTIES OF ZONE 2

	M.p., °C.	$[\alpha]_D$	M_D
Sterol	131	+ 6.4°	+23.9°
Acetate	140	+ 6.0	+28.2
Benzoate	160	+11.9	+58.0

as previously described.[21] A terminal methylene group at this position is known to not greatly influence the optical rotation of the sterol molecule. The modified Liebermann–Burchard reaction confirmed the presence of the double bond in the 7-position.[5] It has previously been reported that the C_7-double bond takes up 2 moles of perbenzoic acid, under the conditions employed, and as expected the new sterol consumes a total of 3 moles of oxygen.[5] Catalytic reduction of the sterol with Adams catalyst in glacial acetic acid resulted in the formation of a $\Delta^{8(14)}$-sterol and the uptake of one mole of hydrogen. The 8(14) double bond is readily characterized by means of the modified Liebermann–Burchard reaction and the "reduction" product of zone 2 sterol was thus established to have an 8(14)-double bond.[5] The sterol was confirmed to be in the C_{28}-series by its molecular weight which was determined from the saponification equivalent of the acetate under carefully controlled conditions.

Experimental[22]

Preparation of Crude Sterols.—The sterol content was determined on 755 g. of macerated starfish. After moisture, 76.8%, had been determined on an aliquot, the material was extracted by shaking with 3 batches of acetone. The oil, 4.49 g. (0.60%), was treated with acetone to remove phospholipids, 1.00 g. (22.3%), and the residue yielded 1.01 g. (22.5%) of non-saponifiable material. When this was chromatographed on an alumina column, 328 mg. (32.5%)

(20) D. H. R. Barton, *J. Chem. Soc.*, 813 (1945).

(21) L. A. Cort, *et al.*, *ibid.*, 3713 (1954).

(22) Melting points are uncorrected. Optical rotations were measured by means of a Rudolph precision polarimeter. Infrared spectra were recorded with a Beckman IR 4 instrument.

of crude sterol was obtained. In one experiment the gonads and digestive organs of mixed male and female animals were determined separately and were found to contain 88.4% of the total sterols.

Chromatography.—The azoyl esters of the crude sterol mixture were chromatographed on silicic acid–Celite columns with the use of 5.5:1 Skellysolve C–benzene developer as previously described.[13] Five zones developed. A column of double length (50 cm.) was necessary to completely separate zones 3 and 4 from each other. Starting with the uppermost zone the composition was: zone 1, 9.0%; zone 2, 19.2%; zone 3, 29.5%; zone 4, 39.8%; and zone 5, 2.5%.

Zone 2 Sterol and Derivatives.—The azoyl ester was crystallized from benzene–ethanol and hydrolyzed. The acetate was prepared and crystallized once from aq. ethanol. The acetate contained 0.3% of provitamin D as calculated from the ultraviolet spectrum. This was removed with maleic anhydride as previously described. After several crystallizations from aq. ethanol, the acetate melted at 140°C., $[\alpha]^{22}D + 6.4°$ (*c* 3.2 in $CHCl_3$).

Anal. Calcd. for $C_{30}H_{48}O_2$: C, 81.76; H, 10.98. Found: C, 81.80; H, 10.66.

The acetate was hydrolyzed and the free sterol crystallized from aq. ethanol, m.p. 131°, $[\alpha]^{22}D +6.0°$ (*c* 2.0 in $CHCl_3$).

Anal. Calcd. for $C_{28}H_{46}O$: C, 84.35; H, 11.63. Found: C, 84.28; H, 11.82.

The benzoate was crystallized from acetone, m.p. 160°, $[\alpha]^{22}D +11.9°$ (*c* 1.4 in $CHCl_3$); molecular rotational differences: 7,24-ergostadien-3β-ol, Δ^{Ac} +4, Δ^{Bz} + 34; Δ^7-stenols, $\Delta^{Ac} -15 \pm 15$, $\Delta^{Bz} +20 \pm 14$.[20]

Anal. Calcd. for $C_{34}H_{48}O_2$: C, 83.55; H, 9.90. Found: C, 83.64; H, 9.86.

Saponification Equivalent.—Zone 2 acetate, 257.766 mg., was saponified in 5.00 ml. of 0.04736 *N* alcoholic NaOH for 1 hour and the residual alkali was back-titrated with 17.95 ml. of 0.09968 *N* HCl. A correction factor, 0.9935, was employed which was derived from several determinations of the molecular weight of highly purified cholesteryl acetate.

Anal. Calcd. for $C_{30}H_{48}O_2$: mol. wt., 440.7. Found: mol. wt., 442.6.

Perbenzoic Acid Titration.—On standing for five days at −5° in an excess of perbenzoic acid in $CHCl_3$, 19.006 mg. of zone 2 sterol consumed 2.11 mg. of oxygen. The theoretical uptake for three atoms of oxygen is 2.07 mg.

Ozonolysis.—Zone 1 sterol (100 mg.) was suspended in 4 ml. of acetaldehyde-free acetic anhydride–acetic acid (4:1) and cooled in an ice-bath. Ozonated oxygen was passed through the suspension at a rate of 10 mg. ozone/l. of oxygen until all of the solid was dissolved. Water and zinc dust were then added and the mixture was heated in a water-bath in order to decompose the ozonide. The mixture was steam distilled into a 0.5% dimedone solution. The *p*H of the solution was adjusted to 5.8 and the precipitate collected.

The dimedone derivative weighed 52.8 mg. (71.8% of the theory for one methylene group), recrystallized m.p. 189°, mixed m.p. 190° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.91; H, 8.49.

The filtrate from the dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazone solution, which was then extracted with ethyl acetate. No hydrazone was obtained.

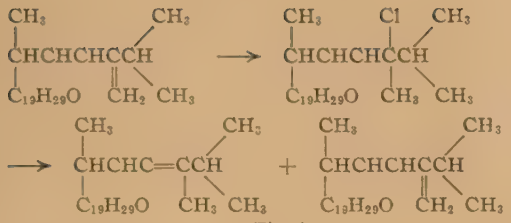

Fig. 1.

Rearrangement with HCl.—Zone 2 acetate (430 mg.) was dissolved in 5 ml. of $CHCl_3$ and HCl gas was led through the solution for 6 hours. The solvent was evaporated and the residue was boiled for 10 hours with 6 ml. of acetic anhydride. The solution was then diluted with water, the precipitate collected and chromatographed on an alumina column, from which 380 mg. of material was recovered.

The product from the column was suspended in 10 ml. of acetic anhydride–acetic acid (4:1) and ozonized as described above. The dimedone derivative weighed 71 mg. (28.3% calculated as acetate), m.p. 189° after crystallization, mixed m.p. 189° with authentic formaldehyde dimedone.

Anal. Calcd. for $C_{17}H_{24}O_4$: C, 69.82; H, 8.27. Found: C, 69.85; H, 8.89.

The remaining dimedone solution was steam distilled into a 2,4-dinitrophenylhydrazone solution. The solution was extracted with ethyl acetate and the evaporation residue chromatographed on a silicic acid–Celite column (2:1). Skellysolve C–benzene (2:1) moved a band which was eluted (66 mg. representing 28.7% calculated as acetate) and crystallized from aq. methanol, m.p. 123°, mixed m.p. 123° with authentic methyl isopropyl ketone.

Anal. Calcd. for $C_{11}H_{14}O_4N_4$: C, 49.62; H, 5.30. Calcd. for $C_{12}H_{16}O_4N_4$: C, 51.42; H, 5.75. Found: C, 49.68; H, 5.26.

The 2,4-dinitrophenylhydrazone of the ozonolysis product and of methyl isopropyl ketone had identical R_f values on a descending chromatogram run for 2.5 days in isopropyl alcohol–water (1:1) on Whatman No. 1 filter paper.[23] Ethyl isopropyl ketone, isovaleraldehyde, methyl isopropyl acetaldehyde and ethyl isopropyl acetaldehyde had lower R_f values.

Acknowledgment.—Mr. A. P. Ronald of our microanalytical section performed many of the analyses and recorded the infrared spectra.

(23) R. B. Seligman and M. D. Edmonds, *Chemistry & Industry*, 1406 (1955).

Vancouver 2, B. C., Canada

VARIATIONS IN THE SODIUM AND POTASSIUM CONTENT OF THE MUSCLE TISSUE OF PACIFIC SALMON WITH PARTICULAR REFERENCE TO MIGRATION[1]

ROBERT A. MACLEOD, R. E. E. JONAS, AND J. R. MCBRIDE

Abstract

Further confirmation was obtained of the observation made previously that the Na^+ and K^+ levels of the muscle tissue of spring salmon (*Oncorhynchus tschawytscha*) on the Pacific coast appear to vary with the size of the fish and, in the case of small fish, with the season of the year. Small fish (3 to 5 lb) caught in regions of relatively constant salinity had higher Na^+ and lower K^+ concentrations in the spring and summer than in fall and winter. Under the same conditions the levels of Na^+ and K^+ in the flesh of larger fish (9 to 10 lb) did not vary.

When spring salmon were sampled along the route of their spawning migration up the Fraser River it was found that at the mouth of the river the Na^+ level was less than in fish at sea. Further up the river, Na^+ had dropped to less than one-half of the value at sea. At the spawning grounds in three groups out of four examined, Na^+ rose again to levels the same as or above those prevailing at sea. In general, a drop in Na^+ was accompanied by a rise in K^+ and vice versa, though at the spawning ground the drop in K^+ considerably exceeded the rise in Na^+.

In the case of sockeye salmon (*Oncorhynchus nerka*) heading up river, a lowered Na^+ and elevated K^+ occurred in the muscle tissue of the fish while they were still in salt water. After the fish had entered fresh water the ion levels were restored and maintained at levels similar to those normally prevailing at sea. At or near the spawning ground a marked rise in Na^+ as well as a drop in K^+ occurred. Evidence was obtained that this change occurred first in the male of the species.

The flesh of juvenile coho salmon (*Oncorhynchus kisutch*) migrating to the sea was found to contain less Na^+ and more K^+ than mature fish of the same species caught at sea. Young sockeye in fresh water, however, contained about the same levels of Na^+ and K^+ as the mature fish at sea.

Introduction

Fish which migrate from fresh water to sea water or from sea water to fresh water or both face very special problems in osmoregulation. In sea water the osmotic pressure of the medium is greater than that of the body fluids. To maintain the water and salt balance in sea water, teleost fish drink sea water and excrete excess inorganic ions through the gills and by way of the urine. In fresh water the reverse holds. Excess water is excreted by an increased urine flow and salts are conserved. For a review see Black (4).

It has been found that adult salmon returning to fresh water exhibit a slight decrease in osmotic concentration of the blood (2,9). At the spawning grounds this decrease amounted to 17% of the concentration at sea. Eels (*Anguilla* spp.) migrate as adults from continental fresh waters to spawn in the Atlantic Ocean. Loss of body salts has been observed in these fish prior to entering the sea (5). Whether these changes occur as a result of alterations

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in the salt content of the environment or are produced by hormone effects associated with migration has not been established clearly.

In a previous investigation a survey was made of the Na^+ and K^+ content of the flesh of fish caught in commercial quantities on the coast of British Columbia (12). It was noted that in the case of salmon, considerable variations in the flesh levels of these ions occurred which appeared to depend on the size of the fish and the season when they were caught. It was speculated that the change with season might be related to the approach of the fish to the time of their spawning migration. The present study was undertaken to explore this possibility further. Samples of both spring and sockeye salmon were obtained at various points along the route of their migration up the Fraser River and their flesh levels of Na^+ and K^+ determined. The levels of Na^+ and K^+ in two species of young salmon migrating downstream to the sea have also been obtained. Marked variations in flesh levels of the ions have been observed and factors responsible for these changes considered.

Experimental

Source of Materials

Fish from the sea were obtained directly from fishing boats. Spring salmon (*Oncorhynchus tschawytscha*) caught at various points on the Fraser River were obtained through the kind co-operation both of the Department of Fisheries, Vancouver, and of Dr. D. J. Milne of the Biological Station at Nanaimo. The fish were placed individually in polyethylene bags when caught and the bags were then packed in ice for transport to the laboratory. This arrangement prevented possible leaching of the fish by the melting ice.

Sockeye salmon (*Oncorhynchus nerka*) samples were obtained through the co-operation of the International Pacific Salmon Fisheries Commission. The fish collected in 1956 were transported under the same conditions as those used for the spring salmon. In 1957, however, fish were bled and then frozen in dry ice at the site of capture. Transport of these fish to Vancouver was effected, using a portable deep-freeze unit. All of the samples of sockeye salmon were used as sources of material for other experimental work besides that recorded here. More complete details of the capture and handling of these fish are presented elsewhere (10).

Methods of Sampling and Analysis

All fish were eviscerated and then sampled according to a slight modification of the official A.O.A.C. method. Three slices were removed from each fish, one from behind the head, one from the mid-section, and one from just in front of the tail. The muscle tissue from each slice was separated from the bones and skin, pooled, and homogenized. Aliquots of the homogenized samples were solubilized with nitric acid. Analyses were performed using a flame photometer attachment for a Beckman DU spectrophotometer. Details of the procedures used have been described (12). Fat analyses were performed by the method of Damberg (7).

Results

Fish Caught at Sea

In the course of a previous investigation into the Na^+ and K^+ levels in the flesh of Pacific salmon (12), it was observed that spring salmon in the small size range (2 to 7 lb) had higher Na^+ and lower K^+ levels in the spring of the year than later in the summer. In the summer the levels approached those of fish in a larger size range (7 to 20 lb).

It was decided to reinvestigate this phenomenon for the following reasons. The sea is not a perfectly uniform environment. Areas of sea water near the shore can be diluted by fresh water from rivers. The types of food ingested by fish vary with availability from season to season and from one year to another. Since fish tend to run in schools, fish caught at any one time are not necessarily random samples but may all be representative of a group of fish which have been subjected to similar environmental conditions.

It was felt that if the types of variation observed previously were found to occur in another year, the results could be more readily attributed to physiological changes in the fish rather than to responses of the fish to transitory environmental conditions. Accordingly, fish in two size ranges were caught at different times during 1956 at points along the west coast of Vancouver Island, where the salinity of the sea water is not influenced appreciably by run-off from the Fraser River (13).

TABLE I

EFFECT OF SIZE AND SEASON ON THE Na^+ AND K^+ CONTENT OF THE MUSCLE TISSUE AND TISSUE WATER OF SPRING SALMON CAUGHT AT SEA*

Size range†	Time of catch	No. of fish	Average wt., lb	Na^+ and K^+ in fat-free tissue, meq per kg§		Na^+ and K^+ in tissue water, meq per kg	
				Na^+	K^+	Na^+	K^+
Small	July 20	3	4.94 ± .42‡	28.4 ± 3.0	112.7 ± 1.6	36.4 ± 3.9	144.2 ± 2.0
	Aug. 1	3	3.50 ± .28	23.9 ± 0.6	114.0 ± 1.1	30.8 ± 0.7	147.3 ± 1.4
	Dec. 1	3	4.94 ± .70	19.1 ± .32	127.2 ± 0.8	24.4 ± 0.4	162.2 ± 1.0
Medium	July 20	3	10.40 ± .47	19.6 ± 1.5	123.9 ± 4.0	26.0 ± 2.0	164.7 ± 5.3
	Aug. 1	3	9.31 ± .67	19.1 ± 1.0	120.7 ± 1.4	24.9 ± 1.3	157.2 ± 1.8
	Dec. 1	3	10.00 ± .67	19.6 ± 2.5	125.4 ± 5.7	25.3 ± 3.2	162.1 ± 7.4

*West coast of Vancouver Island.
†Small, 2–7 lb; medium, 7–20 lb; large, 20 lb and up.
‡Deviations shown are average deviations of the mean.
§meq per kg = milliequivalents per kilogram.

The results in Table I show that small fish caught in July had significantly higher Na^+ and lower K^+ levels than those caught in December ($P < .02$ in each case). An intermediate value was recorded in August, suggesting a downward trend for Na^+ and an upward one for K^+ between July and December. In December, the concentrations found were the same as those which had prevailed in fish in the medium size range over the whole period. In the previous study (12) the drop in Na^+ and rise in K^+ had been found to occur

between June and July. In the present case the change in levels did not begin until after the July sample was taken.

Since the reasons for the variation in the Na^+ and K^+ content of the fish with size were obscure and since the factors mentioned above could introduce so many sampling problems, other fish in two size ranges were analyzed for their Na^+ and K^+ concentrations. In this case the fish were caught during May and June since the previous study (12) had indicated that variations in the ion concentration with size could most likely be expected at this season. Nine fish in the small size range and 12 in the large were sampled and analyzed over the 2-month period. The results (Table II) show that the differences noted previously between large and small fish occurred again in this experiment. Larger fish almost invariably contain more fat in the muscle tissue than small ones as the results in Table II show. That this is not the factor

TABLE II

COMPARISON OF Na^+ AND K^+ LEVELS IN MUSCLE TISSUE AND TISSUE WATER OF SPRING SALMON IN TWO SIZE RANGES CAUGHT AT SEA*

	Size range		Significance of differences
	Small	Large	
Tissue levels‡			
Na^+, meq/kg	23.04 ± .76†	17.21 ± .68	$P < .001$
K^+, meq/kg	115.0 ± 2.9	128.1 ± 2.9	$P < .001$
Tissue water levels			
Na^+, meq/kg	28.42 ± 3.17	22.38 ± .82	$P = .05$
K^+, meq/kg	141.8 ± 9.5	166.5 ± 4.6	$P = .02$
Weight of fish, lb	4.92 ± .52	20.37 ± 1.09	
Fat content, %	2.84 ± .24	7.56 ± 0.90	
No. of fish analyzed	9	12	

*All fish caught in May and June.
†All deviations reported are standard error of the mean.
‡Based on fat-free muscle tissue.

responsible for the variation in ion levels is evident from the fact that all calculations of muscle levels of ions were made on the basis of fat-free tissue.

The findings presented here and observed previously (12) indicate that at certain times of the year the muscle tissue of small spring salmon can be expected to contain more Na^+ and less K^+ than large ones.

Fish Migrating Upstream

Salmon often travel hundreds of miles up rivers to their spawning grounds. On these journeys no food is consumed. It was of interest to know how well flesh levels of Na^+ and K^+ were maintained during upstream migration. Accordingly, arrangements were made to obtain spring salmon caught at various points on the Fraser River and at the spawning grounds.

Fresh water from the Fraser River reduces the salinity of the sea water for a considerable distance from the river mouth (13). Two groups of fish

were caught at the mouth of the Fraser and their flesh concentrations of Na^+ and K^+ compared with values obtained on fish of the same size caught at approximately the same time at a point where normal ocean salinities prevail. The results show (Table III) that the fish at the river mouth had lower Na^+ and higher K^+ concentrations than occurred in fish at sea.

At the next point taken in the course of upstream migration, a point 90 miles from the sea, the Na^+ concentration in the flesh had dropped to nearly half that at sea, while the K^+ level, except in the case of the one fish sampled, was much the same as the concentration at sea.

Fish from three different spawning grounds were sampled. Two of these, spawning grounds I and II, were 190 and 280 miles respectively from the mouth of the Fraser River, while the third was only a comparatively short distance, 60 miles, upstream. At spawning grounds I and II the Na^+ concentration had risen to levels which were even higher than those in fish at sea, while K^+ had dropped very considerably. As can be seen from the table, the changes recorded appear to be relatively independent of the size of the fish.

The fish on spawning ground III represented a later run of spring salmon. The results obtained here were compared with values from fish caught at sea at approximately the same time. At this spawning ground a random sampling of eight fish gave two groups of four which differed markedly from one another with respect to flesh Na^+ levels. In group I the muscle Na^+ concentrations were the same or somewhat higher than those of fish at sea. In group II, the low Na^+ concentrations characteristic of fish which have just recently entered fresh water were recorded. The reasons for the differences in the two groups of fish at this third spawning ground are obscure. All eight fish involved had spawned but were alive when caught. There was about an equal distribution of males and females in each group. There was no obvious correlation of the results with size.

The analysis for protein shows that the muscle tissue of the spawned fish contained only half as much protein as that of fish at sea. This indicates that since the fish do not eat, body protein plays a major role in providing energy for migration as well as substrate for gonad development. Such an observation has been made previously (9). The loss of muscle protein and hence of muscle tissue cells could account for the drop in muscle K^+ concentration. If cells are being replaced by extracellular water, one might also expect a rise in tissue Na^+. That this is not the only explanation for the increase in tissue Na^+ at the spawning ground, however, is indicated by the fact that both groups of fish at spawning ground III have similarly low muscle protein levels, but considerably different tissue Na^+ concentrations.

Mature sockeye salmon differ from spring salmon in being of a very uniform size (5 to 7 lb). A number of different runs of this species of salmon each consisting of a relatively pure race of fish having a particular spawning ground as their goal can be distinguished each year on the Fraser River (11). One of these runs was sampled in 1956. The results are recorded in Table IV. In this study three fish were obtained at each of the points shown. Since the

TABLE III

COMPARISON OF Na^+ AND K^+ LEVELS IN MUSCLE TISSUE AND TISSUE WATER OF SPRING SALMON CAUGHT AT VARIOUS POINTS ON THE COURSE OF THEIR UPSTREAM MIGRATION

Location	No. of fish	Size	Na^+ and K^+ in fat-free tissue, meq/kg		Na^+ and K^+ in tissue water, meq/kg		H_2O, %	Protein, %
			Na^+	K^+	Na^+	K^+		
At sea*	3	Medium	19.1 ± 0.7**	120.7 ± .8	24.9 ± .91	157.2 ± 1.1	71.3	—
River mouth†	3	Medium	15.4 ± 0.3	134.0 ± 4.1	21.1 ± .45	183.0 ± 5.6	68.1	—
	2	Medium	14.0 ± 3.3	128.7 ± 4.6	18.4 ± 4.3	170.0 ± 6.2	70.7	—
Upriver 90 miles‡	1	Medium	6.76	103.6	8.4	129.5	77.2	—
Upriver 90 miles	2	Small	9.62 ± .51	122.7 ± 1.4	12.6 ± .67	160.5 ± 1.8	71.9	—
Spawning ground I§	3	Small	22.7 ± 0.3	109.3 ± 0.8	28.5 ± 0.4	137.5 ± 1.0	76.1	—
Spawning ground II‖	4	Small	23.6 ± 3.9	86.3 ± 7.0	28.0 ± 4.6	102.3 ± 8.3	83.2	—
Spawning ground II	4	Medium	26.2 ± 4.4	87.0 ± 8.0	31.6 ± 5.3	105.0 ± 9.7	81.8	—
At sea*	1	Small	19.0	127.9	24.3	162.9	73.5	19.5
	1	Medium	19.7	126.3	25.1	160.8	74.6	19.2
Spawning ground III¶								
Group I	4	—	21.0 ± 2.0	80.8 ± 2.3	23.7 ± 2.3	91.1 ± 3.3	88.3 ± .7	10.3 ± .9
Group II	4	—	11.6 ± 1.4	89.3 ± 6.3	13.1 ± 1.6	101.3 ± 7.3	87.7 ± 1.3	11.2 ± 1.0

*West coast of Vancouver Island; †Fraser River; ‡Hope, B.C.; §Seton Creek, up the Fraser River 190 miles.
‖Adams River, up the Fraser River 280 miles; ¶Harrison River, up the Fraser River 60 miles.
**Deviations reported are average deviations of the mean.

TABLE IV

CHANGES IN THE LEVEL OF Na^+ AND K^+ IN THE MUSCLE TISSUE AND TISSUE WATER AND IN THE WATER, FAT, AND PROTEIN CONTENT OF THE MUSCLE TISSUE OF SOCKEYE SALMON DURING UPSTREAM MIGRATION (STUART RUN, FRASER RIVER, 1956)

Location	Miles upriver*	Na^+ and K^+ in fat-free tissue, meq/kg		Na^+ and K^+ in tissue water, meq/kg		H_2O, %	Fat, %	Protein, %
		Na^+	K^+	Na^+	K^+			
San Juan Is.	At sea	19.49 ± 1.49§	120.0 ± 0.7	26.30 ± 1.94	162.0 ± 1.4	69	6.2	23.1
Lummi Island	135†	13.48 ± 0.59	128.5 ± 0.7	18.16 ± 0.76	173.2 ± 1.2	69	6.6	22.7
Albion	215	17.82 ± 0.76	113.7 ± 1.4	23.79 ± 1.05	151.8 ± 1.7	71	5.2	22.4
Hell's Gate	305	17.54 ± 1.31	118.3 ± 2.5	23.13 ± 1.65	156.0 ± 3.8	73	3.9	22.2
Lillooet	385	17.05 ± 2.08	113.7 ± 1.5	22.52 ± 2.52	150.3 ± 3.3	73	3.7	21.5
Soda Creek	515	18.51 ± 0.80	110.0 ± 3.2	24.18 ± 1.00	143.7 ± 4.3	75	2.5	21.4
Fort St. James	775	26.36 ± 4.10	96.1 ± 2.7	32.81 ± 5.04	119.8 ± 4.2	79	1.6	18.4
Forfar Creek‡	850	31.54 ± 2.06	94.2 ± 0.9	39.55 ± 2.85	118.0 ± 0.6	79	1.3	19.2

*Up the Fraser River from San Juan Island.
†This point is still in sea water.
‡Spawning ground.
§Deviations recorded are average deviations of the mean.

fish were to be used in other investigations as well, one side only of each was taken for analysis. Sampling was conducted in the usual way, however, by pooling the skin-free, bone-free muscle tissue from three slices from each fish. It can be seen from the results that in the case of sockeye salmon the drop in Na^+ occurred even before the fish entered fresh water. For a good part of the distance up the river the Na^+ levels were very nearly the same as those of the fish at sea. Over the last one-third of the journey the Na^+ levels rose until, at the spawning grounds, they were 75% higher than the values at sea. During this latter part of the journey a progressive decline in K^+ occurred.

In view of the difference in Na^+ concentration with sex at the spawning ground which will be described in connection with the next table (Table V), it is of interest to consider the sex of the fish analyzed in this experiment. The three fish sampled at Fort St. James (Table IV) consisted of two females and one male. The male fish had the high tissue Na^+ concentration of the fish at Forfar Creek while the two female fish had levels similar to those prevailing in the fish at Soda Creek. The three fish at Forfar Creek spawning ground also consisted of two female and one male fish. In this case, however, the two female fish had the same high tissue Na^+ concentrations as the male.

It should also be noted that the percentage rise in tissue Na^+ level of the sockeye salmon at the spawning ground was considerably greater than was observed in the case of the spring salmon. On the other hand, the protein loss from the muscle tissue of the sockeye salmon was very much less than occurred in the spring salmon. These species differences lend further support to the conclusion reached previously that it is unlikely that the rise in tissue Na^+ at the spawning ground is due primarily to replacement of muscle tissue cells by extracellular water.

Samples of the same run of sockeye were obtained the following year (1957). In this case a much larger number of fish were sampled. Four groups of males and four groups of females, each group containing 8 to 10 fish, were obtained from each of two points, Lillooet and the spawning ground, Forfar Creek. Each fish was sampled by removing a 1-in. cube of flesh from left side of the back immediately ahead of the dorsal fin. These fish differed also from those studied previously in that they had been bled prior to sampling. Comparison of the Na^+ values at the two points (Table V) show that the greatest rise in Na^+ occurred in the males, though a significant rise in this ion (significance of difference, $P < .05$) also occurred in the females. In both cases the K^+ levels dropped considerably.

Young Salmon in Fresh Water

Because of the marked variations in the concentration of Na^+ and K^+ in the flesh of adult salmon it was of interest to know what levels existed in young salmon found in fresh water. Juvenile sockeye and coho salmon (*Oncorhynchus kisutch*) which were either in the process of migrating downstream or due shortly to begin the trip to the sea were obtained for analysis.

TABLE V

COMPARISON OF THE Na^+ AND K^+ LEVELS IN THE MUSCLE TISSUE AND TOTAL TISSUE WATER OF MALE AND FEMALE SOCKEYE SALMON AT TWO POINTS ON THE FRASER RIVER STUART RUN (1957)

Location of catch	Miles upstream	Sex	Na^+ and K^+ in fat-free tissue,* meq/kg		Na^+ and K^+ in tissue water, meq/kg	
			Na^+	K^+	Na^+	K^+
Lillooet	385	Male	12.98 ± .21†	118.6 ± 0.8	17.26 ± .21	157.8 ± 1.4
		Female	13.42 ± .26	117.2 ± 1.2	17.73 ± .31	154.8 ± 1.3
Forfar Creek (spawning ground)	850	Male	30.98 ± 2.69	102.0 ± 2.7	38.63 ± 3.35	127.2 ± 2.1
		Female	16.16 ± 0.39	111.6 ± 0.9	20.26 ± 0.46	140.0 ± 1.8

*Based on fat-free skeletal muscle tissue. In this experiment only, the fish were bled immediately after capture.
†Deviations recorded are standard errors of the mean.

TABLE VI

Na^+ AND K^+ IN THE MUSCLE TISSUE AND TOTAL TISSUE WATER OF JUVENILE SALMON OBTAINED FROM FRESH WATER

Species	Source*	No. of samples†	Na^+ and K^+ in fat-free tissue, meq/kg		Na^+ and K^+ in tissue water, meq/kg	
			Na^+	K^+	Na^+	K^+
Coho	Capilano River	4	10.34 ± .93‡	123.0 ± 1.5	12.59 ± 1.13	149.7 ± 1.9
Coho	Cultus Lake	3	16.19 ± 0.18	145.6 ± 1.3	20.43 ± .22	183.7 ± 1.6
Sockeye	Cultus Lake	3	24.56 ± .43	106.9 ± 2.1	–	–

*Analysis of H_2O samples: Capilano River, Na^+ .039, K^+ .026 meq/kg. Cultus Lake Na^+ .248, K^+ .026 meq/kg; by comparison, sea water contains approximately Na^+ 457, K^+ 9.75 meq/kg.
†Because of the small size of each fish, each sample consisted of flesh obtained by pooling skin- and bone-free portions of 10 to 15 fish.
‡Deviations recorded are average deviations of the mean.

Table VI shows that the flesh of coho salmon from one source had extremely low Na^+ levels, while those from another were considerably higher, though still well below the average for mature fish of this species at sea (23–24 meq/kg) (12). Corresponding K^+ levels were above the average for these fish at sea. In the case of the sockeye, the Na^+ value obtained was even slightly higher while K^+ was about the same as the averages for mature fish of this species at sea. That these levels bear no obvious relation to the content of Na^+ and K^+ in the water in which the fish were caught is apparent from the results of analyses which are recorded beneath Table VI.

Discussion

The findings presented here and observed previously (12) indicate that at certain times of the year the muscle tissue of small spring salmon contains more Na^+ and less K^+ than that of larger fish. The reason for this is obscure. Barlow and Manery (1) found that the muscle tissue of young chicks contained more Na^+ and less K^+ than that of adults and concluded that this change with age was due to the displacement of extracellular fluid by cells. Since

small salmon are in most cases also young salmon, a similar situation could conceivably exist in the case of small and large fish. Since the elevated Na^+ and lowered K^+ in small salmon occurred only at certain seasons of the year, however, it is difficult to attribute to maturation the establishment at other seasons of levels prevailing in larger fish.

During upstream migration the changes in Na^+ and K^+ were somewhat different in the two species of salmon examined. In the case of sockeye, a lowered Na^+ and elevated K^+ occurred in fish still in salt water (Lummi Island), suggesting that in this species the initial change observed may not be a consequence of the fish entering fresh water but a reflection of a change in hormone levels associated with migration. After this preliminary change, even though the fish entered fresh water, the ion levels were restored almost to the levels which normally prevailed at sea. These concentrations were then maintained until the spawning grounds were either reached or closely approached.

Whether, in the case of spring salmon, ion changes occurred in the tissues before or after the fish had entered fresh water cannot be stated. There was not the same opportunity to follow a group of spring salmon from salt water to fresh water as existed with the run of sockeye salmon. Once the fish were in fresh water, however, much more drastic reductions in Na^+ level occurred in spring salmon than sockeye and these obtained for much longer distances up the river.

At the spawning grounds an unusual and unexpected situation prevailed. At all of the spawning grounds tested and for both species of salmon, fish were found which had elevated Na^+ and lowered K^+ concentrations in the muscle tissue. Although this would appear to be the general trend of ion changes at the spawning ground, two exceptions to this pattern were noted. One group of spring salmon at one of the spawning grounds showed no elevation in tissue Na^+ though this group appeared to be in exactly the same physiological state as another group which did display the high Na^+ concentration. For sockeye salmon, in the 1957 experiment, the marked rise in Na^+ level occurred only in the male.

No explanation can be offered for the variation in tissue Na^+ concentration in spring salmon at the third spawning ground. For the sockeye salmon there is an apparent discrepancy between the results of the 1956 and 1957 experiments in regard to the rise in tissue Na^+ in the female fish at the spawning ground. In the 1956 experiment both male and female fish showed high tissue Na^+ levels at the spawning ground while in 1957 the steep rise was recorded only in the male. A possible explanation for this discrepancy lies in the fact that this particular run of fish does not spawn immediately after the fish have reached the spawning grounds but rather about a week after the fish have arrived. During this week, the final stages in gonad development apparently occur. If the rise in tissue Na^+ takes place in male fish before it occurs in female fish and if the runs in the two successive years were not sampled at the spawning ground at a time when the fish were in exactly the

same stage of physiological development, the apparent discrepancy in results could occur. In support of this possibility is the indication in the 1956 sockeye experiment that the rise in tissue Na^+ occurred in the male fish even before the spawning ground was reached, though insufficient numbers of male and female fish were included in the 1956 samples to permit any definite statement to be made. The fact that a small but significant rise in muscle Na^+ had occurred in the female fish at sampling time in the 1957 experiment lends further support to the explanation offered. In addition, in the 1957 study, the last sample was taken immediately after the fish had arrived at the spawning ground, so that approximately a week would have remained for further changes in the tissue Na^+ concentration of the female to have taken place.

The drop in tissue K^+ concentrations in spring salmon at the spawning grounds is much greater than for sockeye salmon and this species difference is roughly in proportion to the much lower muscle protein concentration in the spring salmon. A loss of muscle tissue cells through starvation could thus be largely responsible for the loss in tissue K^+. That it is not entirely responsible is suggested by the fact that the drop in tissue K^+ in sockeye salmon in the 1957 experiment, Table V, was greater in the male fish, though protein losses from the muscle have been found to be appreciably greater in the female fish at this point owing to the fact that in the latter the gonads developed represent a much larger proportion of the total body weight (10).

That the loss of muscle tissue cells and its replacement by extracellular water is unlikely to be responsible for the marked rise in tissue Na^+ observed at the spawning grounds has already been considered at various points in the text.

In all cases observed at the spawning ground the drop in K^+ exceeded the rise in Na^+ and the sum of the Na^+ and K^+ concentrations in the tissue water was less than was found at sea. Since there is an increased percentage of water in the flesh of fish at the spawning ground, it might at first appear that the lowered electrolyte concentration was due to dilution. Another investigation has shown, however, that for the sockeye salmon there is only an approximately 10% increase in the volume of muscle tissue water in the male and an actual decrease in the female during migration (10). The increased percentage of water recorded in Table IV then is due largely to a reduction in the amount of protein and fat in the tissue rather than to a gain in tissue water.

The sharp rise in Na^+ and at least part of the drop in K^+ which has been observed to occur in the tissues of the two species of salmon at the end of the migration could conceivably be due to heightened hormone activity associated with the final stages of sexual development before spawning. A similar rise in muscle Na^+ and drop in K^+ occurred in brown trout as a result of an injection of 11-deoxycorticosteroids (6) and it is the same type of response which has been obtained from an injection of these hormones in mammals. So far, however, this particular group of hormones has not been shown to occur in fish. In elasmo-branchs, changes in interrenal activity have been

detected during the sexual cycle (8) though in this group of fish cortical steroids appear to affect carbohydrate rather than mineral metabolism (3).

The Na^+ and K^+ levels found in the flesh of coho and sockeye fingerlings migrating to sea indicate that the sockeye differ from the coho in having flesh levels of the ions very much like those of mature fish of the same species at sea. It may be of significance in this connection that sockeye can, if necessary, spend all their life in fresh water while coho must migrate to the sea to survive.

The results recorded here show that both at sea and in fresh water wide variations in flesh levels of Na^+ and K^+ occur in two species of Pacific salmon. They also indicate that such factors as size of the fish, season of the year, salinity of the water, sex of the fish, and probably also hormone interrelationships, may determine the concentrations of these ions in the flesh. To understand better the mechanism of the changes which occur, considerably more information about such factors as ion changes in the blood of the fish, distribution of ions between intracellular and extracellular water in the flesh, hormone changes, and the influence of these on electrolyte metabolism in fish are required. Since most of the phenomena investigated here cannot be reproduced in the laboratory it is necessary to obtain samples far from normal laboratory facilities. The only samples on which reliable Na^+ and K^+ determinations could be made with present facilities were samples of whole flesh. When mobile laboratory equipment permitting studies at the site of capture of the fish becomes available, a more searching analysis of some of the factors which have been brought to light in this investigation will be made.

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OBSERVATIONS ON MARINE BIRDS OFF SOUTHWESTERN NOVA SCOTIA

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The principal accounts of the birds of Nova Scotia (Downs, 1888; Allen, 1916; Tufts, 1956) give few details on the marine birds of that province. Accordingly, our records of marine birds observed in the summer of 1957 off the southwestern coast of Nova Scotia may be of some interest.

Most of our observations were made at sea within a 10-mile radius of Lockeport, Shelburne County, Nova Scotia, during two periods: May 27 to June 24 and August 16 to 23. On each of 28 days during these two periods we spent at least four hours at sea. Most of the time at sea was spent within three miles of land. We went beyond this limit on eight days: May 27, 28 and 30; June 3, 19 and 21; August 16 and 22. In the spring our observations were greatly restricted by the presence of fog in the Lockeport area. We encountered thick fog on 7 of the 21 days spent at sea in May and June. There was no fog during our week in August at Lockeport.

Additional observations were made on a trip by sea from St. Andrews, N.B., to Lockeport, N.S., on August 15 and 16. The course followed on this trip took us past Deer I., N.B., and Campobello I., N.B., then along the east coast of Grand Manan I. and across the Bay of Fundy to Freeport, N.S. From Freeport we crossed St. Mary Bay to Meteghan, N.S., where we spent the night of August 15. The following day we sailed from Meteghan around Cape Sable to Lockeport. Dense fog was encountered between Yarmouth, N.S., and Cape Sable and no observations were made on this part of the journey.

All observations were made incidental to the author's duties on the M. B. *Mallotus* on which he was employed as a fisheries biologist by the Fisheries Research Board of Canada, St. Andrews, N.B. Clarence J. Bayers was the captain of the *Mallotus*. It is a pleasure to acknowledge his interest and assistance. Some records of interest made by Bayers when the author was no longer with the vessel are included.

For most species seen in the Lockeport area, the number of days on which the species was observed and, in parentheses, an approximate total number of individuals seen are noted immediately following the scientific name.

All specimens collected are now in the collection of the Department of Zoology, University of Western Ontario.

Fulmar *Fulmarus glacialis*, 1(3). Three (one collected) were seen on June 19 as they followed the vessel while we were setting trawl about 10 miles south of Lockeport. The visibility on this day was poor, rarely exceeding two miles and much less in the numerous fog patches. The wind was from the

south at 15 mph. Two days later in the same locality no fulmars were seen. This day was clear and windless with a visibility of about 10 miles.

GREATER SHEARWATER *Puffinus gravis*, 9(58). The first was seen and collected on June 6 near the Lockeport whistle buoy, about three miles from the nearest land. Thereafter in June the Greater Shearwater was seen on eight days. The daily numbers exceeded four only on the two occasions after June 6 when we were farther offshore than three miles. On the first of these occasions, June 19, we were setting trawl about 8 to 10 miles south of Lockeport. On this day, 40 Greater and 10 Sooty shearwaters were seen. The birds, particularly the former species, were characteristically fearless and voracious, flying close by the stern of the *Mallotus* to seize or to dive for pieces of herring bait. Three male and two female Greater Shearwaters were collected. In the same area on June 21, when the weather was much different than that on June 19 (see above under Fulmar), only six Greater Shearwaters were noted.

None was seen in the Lockeport area in August. It may be noteworthy that there were no southerly winds or fog during our visit to Lockeport in August.

On August 15 this species was first met about halfway across the Bay of Fundy and in the following hour 11 were seen. Three were seen while crossing St. Mary Bay from Freeport to Meteghan. On the following day three were seen about two miles off Cape St. Mary, N.S.

C. J. Bayers told me that on July 9, 1957, while steaming in a thick fog just south of Meteghan, N.S., he passed for five minutes through a huge raft of Greater and Sooty shearwaters.

SOOTY SHEARWATER *Puffinus griseus*, 24(about 125). This species was, apart from gulls and terns, the most regularly seen sea bird in the Lockeport area. It was first seen on May 27 and almost daily thereafter. Usually only single birds or groups of two or three were seen, but on June 14 a small concentration of 15 to 20 birds was seen about one mile south of Ram I. near Lockeport. These were feeding on small fish along the edges of tide rips. Another time, on June 21, 11 were seen in one flock. Sooty Shearwaters were clearly more littoral in their distribution than Greater Shearwaters; on only one day were the latter more common. It is widely known, however, that Greater Shearwaters are many times the more common species on the offshore fishing banks of Nova Scotia and Newfoundland.

Five specimens were collected in the Lockeport area. The first, one of a pair resting on the water close by Lesser Hope Rock, was taken on June 6 about one mile offshore from the entrance to Port Joli harbor. This bird was very thin, in a condition contrasting markedly with the presence of extensive deposits of fat on three birds taken on June 14 near Ram I. The fifth specimen, taken on August 16 about seven miles southwest of Lockeport, was also very fat.

Sooty Shearwaters were not seen on our crossing of the Bay of Fundy until we were within about 10 miles of the Nova Scotian coast. Following the first observation, 11 birds were seen in the next five miles.

LEACH'S PETREL *Oceanodroma leucorhoa*, 6(26). This petrel was rarely seen in the Lockeport area and then usually when we were more than five miles offshore. The species does, however, breed in this area for we found several nesting burrows on the seaward face of Ram I. (43°41′N, 65°02′W). A single egg being incubated by a male petrel was present in the only burrow that we examined on June 4. It is worthy of note that this breeding station of Leach's Petrel and, as we shall see, of the Black Guillemot is farther south than any breeding stations occupied today by these species in Maine (Palmer, 1949). Bent (1919, 1922) stated that both species nested on Seal I., N.S. (43°24′N). Fisher and Lockley (1954, p. 12) apparently erred in stating that Maine was the southernmost breeding station of these species in the western Atlantic.

Eight Leach's Petrels seen about 10 to 15 miles west of Freeport were the only petrels noted on the trip across the Bay of Fundy on August 15.

GANNET *Morus bassanus*, 9(30). Occasional individuals were seen around Lockeport, especially in May and June when a total of 27 gannets was seen. Four of these appeared to have full adult plumage. Twenty were in the dark juvenile plumage and the remaining three were predominantly white except for dark wings. A Gannet in dark juvenile plumage and two with dark wings and white bodies were seen singly near Lockeport on August 16, 17 and 19.

One near the north end of Grand Manan I. and another about halfway across the Bay of Fundy were the only Gannets noted on August 15.

DOUBLE-CRESTED CORMORANT *Phalacrocorax auritus*, 5(46). This species was seen much less frequently than we had expected. In June it was seen only on three days and on each occasion was very close to shore. The largest concentration was a group of 15 noted at the entrance to Port Joli harbor on June 6. In August we saw one flock of 16 and another of 8 cormorants, presumably of this species, flying at a considerable height along the coast.

COMMON EIDER *Somateria mollissima*, 5(61). This bird, like the previous species, was seen only close to the mainland or to small islets. Small groups containing up to 20 birds of various ages were seen on four occasions in June. An adult male with testes about 4 mm long was collected on June 6 from a group of 20 eiders near the entrance to Port Joli harbor. A female with no obvious enlargement of ova was collected on June 20 from a flock of 12 birds near Gull Rock at Lockeport. In August only two eiders were seen near Lockeport.

RED PHALAROPE *Phalaropus fulicarius*, 2(19). When we arrived at Lockeport in May a few Red Phalaropes were present. On May 28 two flocks of eight birds (same flock twice?) and on May 30 three birds were seen resting on the sea about nine miles southeast of Lockeport. C. J. Bayers told me that phalaropes, known to him as sea geese, had been much more

common in the fortnight preceding my arrival at Lockeport. No Red Phalaropes were seen at Lockeport in August.

We encountered thousands of phalaropes while crossing the Bay of Fundy on August 15. Flocks of phalaropes, most containing between 15 and 30 birds, became particularly numerous about two miles east of White Head I. which lies off the southeast corner of Grand Manan I. For an hour as we steamed south, flock after flock of phalaropes crossed our bow as they flew southwest into a stiff breeze. Most flocks were small but two flocks each contained at least 500 birds. Both Red and Northern phalaropes were present with the latter outnumbering the former by at least 5 to 1. Stragglers of both species were noted until we were about 10 miles off the Nova Scotian coast. Two adult female Red Phalaropes were collected. Each was mostly in winter plumage except for extensive patches of chestnut feathers on the belly.

NORTHERN PHALAROPE *Lobipes lobatus*, 3 (ca. 120). None was seen in the vicinity of Lockeport in May or June. The species was present at Lockeport in August.

We first saw this species on August 15. A flock of seven birds was noted in Grand Manan Channel. Thereafter on the same day many flocks were seen as we skirted the east side of the Grand Manan Archipelago. Like the preceding species the Northern Phalarope was seen most frequently in the 10-mile stretch of sea extending south from White Head I. None was seen on the Nova Scotian side of the Bay of Fundy. No phalaropes were seen on the following day, August 16, until we had rounded Cape Sable to the eastward. At this point, when we were about four miles offshore, we began to meet small numbers of phalaropes. About 60 (all that were seen well were Northern Phalaropes) were observed between Cape Sable and a point three miles south of Cape Roseway. At the latter point, a male Northern Phalarope was collected from a flock of 11 birds. Fifty Northern Phalaropes (three males collected) were seen on August 19 feeding among long rows of drifting seaweed five miles south of Lockeport and four days later, on August 23, three were noted about a quarter of a mile south of Ram I.

PARASITIC JAEGER *Stercorarius parasiticus*. No jaegers were seen in the Lockeport area.

All the jaegers we observed were seen on August 15 on the passage from St. Andrews, N.B., to Meteghan, N.S. Only three were specifically identified. All were pale forms of the Parasitic Jaeger. The first was seen about one mile southeast of Great Duck I. off the east coast of Grand Manan I. The other two were seen in St. Mary Bay about four miles east of Freeport, N.S. These birds were easily identified as they flew south about 50 yards in front of the boat at a height of about 100 feet. Four other jaegers seen on this day were too far off to permit specific identification. One pale bird was seen chasing terns near Wilson's Beach, N.B. A dark jaeger was noted near the north end of Grand Manan I. Two hours later we saw a dark one harrying a flock of 200 phalaropes near White Head I. Finally, a dark jaeger was seen in the Bay of Fundy about 10 miles west of Freeport, N.S.

SKUA *Catharacta skua*, 2(2). On June 7 we were fishing about five miles south of Lockeport. At 10.15 A.M. we saw a large dark bird chasing some Great Black-backed and Herring gulls. Despite the poor visibility in the fog existing at the time, large white flashes on the primaries were clearly evident. The bird was thought to be a Skua. Six hours later while we were fishing about two miles farther inshore, a Skua came close to the stern to pick up fish entrails. The bird was collected. It was an immature female with very worn plumage, possibly that of the first summer. While skinning the bird I noticed that there was almost no subcutaneous fat. Indeed, the bird could well be described as emaciated. Our identification was subsequently confirmed by Mr. L. L. Snyder of the Royal Ontario Museum of Zoology. We sought this confirmation because of the small size of the specimen. Some measurements of this bird were: wing, 364 mm; tail, 137 mm; exposed culmen, 46 mm; tarsus, 64 mm. These dimensions are less than the minima recorded for the North Atlantic race, *C. s. skua*, in Witherby and others (1949, vol. 5, p. 126). The measurements approximate more closely those of *C. s. antarctica* or *C. s. chilensis* as given by Murphy (1936, p. 1011) than those of any other race of Skua discussed by Murphy.

A second Skua was seen clearly at a range of 50 yards on August 22 about eight miles south of Lockeport.

GREAT BLACK-BACKED GULL *Larus marinus*. This species was, apart from the Herring Gull, the most abundant sea bird in the Lockeport area. It was breeding on Ram I. when we landed there on June 4. Downy young at least several days old and eggs well advanced in incubation were found. We had insufficient time to count the number of nests but we guess that there were at least 50 pairs of Great Black-backed Gulls nesting there.

HERRING GULL *Larus argentatus*. This was the most common sea bird around Lockeport, occurring in the hundreds around the wharves in Lockeport harbor, as well as farther offshore. This species, as well as the preceding, was seen commonly on the trip across the Bay of Fundy.

BONAPARTE'S GULL *Larus philadelphia*. This gull was not seen in the vicinity of Lockeport.

On August 15 we saw 150 (mostly adults) off the west side of Deer I., N.B.; 100 off Wilson's Beach, N.B.; and 10 off the north end of Grand Manan I.

COMMON TERN *Sterna hirundo*. Despite the fact that Lockeport lies within the breeding range of the Arctic Tern *S. paradisaea*, and the Roseate Tern *S. dougallii*, the Common Tern was the only tern certainly identified in this area. It was confined almost exclusively to the waters within a mile or two of shore. The Common Tern almost certainly breeds in the Lockeport area. No breeding colonies were found, but on June 20 we saw a dozen or more terns flying with fish in their bills towards some rocky islets near West Head just to the southwest of Lockeport. In August, there were many young terns near Lockeport.

Flocks of two or three hundred terns (species?) were seen on August 15 near Wilson's Beach and again off the north end of Grand Manan I.

RAZORBILL *Alca torda*, 1(1). The only Razorbill seen was collected on June 7 about five miles south of Lockeport. The bird was an adult male in nuptial plumage. The testes were enlarged; the right testis was 10 mm and the left testis 14 mm long.

COMMON MURRE *Uria aalge*, 1(2). Our only record of this species was made on June 24 when we watched two birds in summer plumage about one mile southwest of Ram I.

DOVEKIE *Plautus alle*, 1(1). Only one was seen. It was collected on June 18 one mile south of Ram I. The bird was a female with no obvious enlargement of the ova. Summer plumage had been largely acquired. The remiges, wing coverts and rectrices were faded and badly worn.

BLACK GUILLEMOT *Cepphus grylle*, 6(about 50). This species in the Lockeport area was seen only within two miles of shore. Black Guillemots apparently breed on low cliffs on the landward side of Ram I. We did not look for nests but on several days we saw birds disappearing into crevices in the cliff. A female collected near Ram I. on June 24 had enlarged ova and extensive brood patches. We estimate that there were about 50 guillemots using the cliffs on Ram I. They were commonly seen on the sheltered waters on the landward side of the island, but were rarely seen on the seaward side.

COMMON PUFFIN *Fratercula arctica*, 1(1). An immature male, the only puffin seen, was collected on June 14 five miles south of Lockeport.

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THE GENUS PROROCENTRUM EHRENBERG. MORPHODYNAMICS, PROTOPLASMATIC STRUCTURES, AND TAXONOMY[1]

ADAM BURSA[2]

Abstract

The morphodynamics of *Prorocentrum* species have been studied comparatively in cultures and in plankton. Plankton populations show little morphological variation. In cultures a variety of forms, often similar to other species of *Prorocentrum* which have been described from distant seas, are found. Morphological variation in *Prorocentrum* species depends upon various life phases, individual features of the clones, and various types of the cysts. Formation of morphological aberrants is also affected by aging of cultures. All protoplasmatic structures, including the cell membrane, possess their own particular features, changing in the course of the life cycle. Since both physiological and morphodynamic metabolisms are inseparable in their activities, it is useful to complete taxonomic diagnoses with physiological observations. Five new species are described: *Prorocentrum cordiformis*, *Prorocentrum pomoideum*, *Prorocentrum pyrenoideum*, *Prorocentrum redfieldi*, and *Prorocentrum levantinoides*. Morphodynamics in *Prorocentrum micans* and *Prorocentrum scutellum* was also studied. Most of the observations were made in vivo. Specific morphological differences in form and structure of protoplasmatic organelles were demonstrated in each species concerned. An attempt was made also to base taxonomic diagnoses upon the apical tooth, trichocysts, and membrane structure.

Introduction

The genus *Prorocentrum* Ehrenb. comprises 31 species abundantly represented in the marine plankton of nearly all seas. Like most of the other dinoflagellates, Prorocentridae were studied mostly in the preserved state. In practice very incomplete data were obtained concerning general features of membrane form and structure. It is desirable therefore to know more about the morphological variation of the cell membrane and living protoplasmatic structures. Phytoplankton samples were collected over a great geographical area, and provide a sufficient basis for a comparative approach concerning the morphodynamics of various species. Cultures kindly given to me by Dr. M. Parke of Plymouth were most useful for experiments and study of all details found in this work. Previous observations of *P. micans* by Braarud and Rossavik (7) in cultures made my work easier, and to some extent are in agreement with my results.

These studies were carried out in the Mediterranean Sea from 1943 to 1945, in the English Channel in 1949 and 1950, in the North Atlantic in 1952.

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Phytoplankton collections taken by the *Calanus* Expeditions in 1953–1954 and 1956 were also examined, with no positive results as far as *Prorocentrum* species are concerned.

Materials and Methods

Materials used are as follows:

A. Living and preserved phytoplankton samples collected by the author on the coast of Israel, from June to September, 1943 and 1944.

B. Living and preserved samples collected by the vessel *Gammarus* of the Plymouth Marine Laboratory in August, 1939 and 1950, in the English Channel.

C. Single-celled and mixed cultures inoculated from Knap Buoy outside Plymouth, England, and kindly made available to the author by Dr. M. Parke. Clones *a*, *b*, *c* of *P. micans* were isolated in September 1949, and *Exuviella* sp. No. 18 was collected in June 1949.

D. Collections from Eel Pond and adjacent waters, Woods Hole, U.S.A., collected by the author in July and September 1952.

E. Bacterized cultures of *P. micans* obtained from Dr. L. Provasoli, Haskins Laboratories, N.Y. (originally sent to Dr. Provasoli by Dr. M. Parke in 1952).

F. Phytoplankton samples collected by the author in the Point Barrow area, Alaska, 1954.

G. Phytoplankton samples collected by the *Calanus* Expeditions in 1953 and 1954.

Neutral red and cresyl brilliant blue (0.1 and 0.3% in aqueous solution) were used for staining in vivo. A little drop of solution placed upon the cover slide was dried, then used to cover a drop of medium containing *Prorocentrum* culture. The gradual penetration of cresyl blue into protoplasm assures slow staining of organelles without destruction. Rapid preserving of *Prorocentrum* was obtained with boiling 10% sublimate and Lugols. Gram's iodine, Schweitzer solution, Marine Dafanos, Champy's fluid, and Janus green were used for staining mitochondria and trichocysts.

Geographical Distribution of *Prorocentrum micans* Ehrenb.

The earliest note of *P. micans* was published by Michaelis (43). It was drawn, mentioned, and described by Ehrenberg (23), Bütschli (14), Schütt (59), Kofoid and Swezy (37), Lindemann (39), Lebour (38), Stein (61), Braarud and Rossavik (7), Biecheler (10), Doflein (20), and other authors.

According to Braarud and Rossavik (7) it is reported from nearly all seas and limited in distribution to between 60° N. and 60° S. It is rare in pelagic, and common in neritic and brackish waters. As pointed out by Gaarder and Gran (25) it is one of the most important dinoflagellates in the Oslofjord plankton. Its maximum recorded by Braarud and Rossavik (7) is in late August and September. Optimum salinity lies between 15 and 20 parts per thousand according to Braarud (7). It is often dominant in the coastal waterblooms of California (Allen (1), Galtsoff (26)). It constitutes the main food of

oysters, according to Martin (42) and Schiller (56, 57). Orton (after Lebour (38)) says it is endobiotic in sponges. It was also squeezed from small sponges floating in Plymouth plankton in 1949 by the author (Fig. 48); whether symbiotic or accidental is unknown. In tracing the geographical distribution, it is necessary to compare this organism from various localities. As comparative morphotaxonomy based on original materials practically does not exist, it is possible to compare only descriptions and drawings from previous reports. Differences in shape and structure of *P. micans* have been noted. The drawing of Kofoid and Swezy (37, p. 81) reveals a perforated membrane with a prominent apical tooth, central nucleus, and large and small anterior pusules. Lebour's drawings (38, Pl. I, Fig. 5) show a spherical pusule at the apical tooth base and posterior nucleus. Schütt (59) and Gran and Braarud (30) show *Prorocentrum* possessing an antapical spine, not reported by others. Cytomorphological observations show other differences also. Martin (42) shows two large stigmas and segmented chromatophores. Biecheler (10) shows a large anterior pyrenoid and central nucleus. It is felt that these structural differences are of importance for taxonomic descriptions of *Prorocentrum* species. These features cannot be evaluated without completing observations on the morphological cycle of different species. It is generally accepted that dinoflagellates represent a typically cosmopolitan group. In the present state of our knowledge on the geographic distribution of species it is too early to draw such conclusions, since vast areas of marine waters are still not investigated. Discoveries of Osorio Tafall (45) and of the present study indicate many new species. Patrick (48), discussing a similar problem in geographic distribution of diatoms, quotes Cleve, who wrote that many diatoms are cosmopolitan, occurring in all parts of the world, but that there are, on the other hand, many species and genera which occur only in certain areas and climates. No *Prorocentrum* species occurred in the Arctic Ocean or in eastern Canadian waters during the present extensive investigations. The very rare Prorocentridae in these waters were represented only by *Exuviella* sp. and *Cenchridium* species.

Orientation in Prorocentridae

Prorocentridae occupy an isolated systematic position among other dinoflagellates. *Paleophalacroma* (Schiller (58)) is thought to represent a transitional link between various *Prorocentrum* species and Dinophysiales. The transverse furrow of *Paleophalacroma* is represented by only a narrow horizontal ridge, while the longitudinal furrow is very reduced and without marginal wings (Schiller (57, 58)).

There is some disagreement as to orientation in Prorocentridae. The flagellate end is regarded as anterior, the valves as left and right according to Bütschli (14). The valves are homologous with the epitheca and hypotheca of Peridiniales, the seam represents the girdle, according to Schütt (59, 60). It is much easier to consider the valve with the flagellar pores as being on the ventral side, and the opposite valve as dorsal. As far as the analogies and

homologies found among various genera of living dinoflagellates are concerned, it seems too early to discuss these features without knowledge of the fossil forms from which contemporary phylogenetic lines probably were developed.

Morphological Variation of *Prorocentrum micans* Ehrenb. in the Sea and in Cultures

Our knowledge of form variation, even in common species, is still far from satisfactory, owing to a paucity of descriptions and the fact that they are based mostly on preserved materials. Graham (28) demonstrated that the form and structure of dinoflagellates is still practically unexplored. The investigations of Braarud (3, 4, 5) constitute a step forward in our knowledge of taxonomy, physiology, and morphology. Little is known on autecology of species. Vigorous growth in culture conditions not existing in the sea shows high adaptability of many forms (36). Woloszynska (65) makes the Baltic biotope responsible for producing some characteristic Baltic forms. According to Paulsen (49, 50), the great variability among certain groups of the marine flagellates is mainly caused by the absence of the sexual phase in their reproduction. There is no doubt that both arguments are correct, but each may apply to only a certain group of species.

Experiments carried out by Braarud (3, 5), Braarud and Pappas (6), and Braarud and Rossavik (7) make it possible to distinguish three different types of morphological variation in dinoflagellates.

(*a*) *Goniaulax tamarensis* in two clones shows definite morphological difference in spite of the fact that the cultures were kept under the same conditions (Braarud (3, 4)).

(*b*) *Peridinium trochoideum* shows no form variation after 5 years in cultures in various salinities, temperatures, and nutrient conditions.

(*c*) *Prorocentrum micans*, a "good species in the sea", produces many aberrants in cultures (Braarud and Rossavik (7)).

The morphological variation in *P. micans* is shown in Figs. 1–22. Three clones of *P. micans* cultured in the Plymouth Laboratory were analyzed from the morphological point of view. Over 3000 cells from plankton were also examined, to obtain information on variations between cultures and the sea. Though many aberrants were recorded, only the more characteristic forms were drawn or described, and the intermediate forms were omitted. The analysis of such a supposedly simple organism as *Prorocentrum* reveals so many details that it is almost impossible to deal with them by morpho-biometrical and statistical methods. However, it is possible to conclude that some clone possessed its own small morphological variation, expressed in the number of membrane pores and their features, and in the shape and structure of the apical tooth. Some rare aberrants were only once observed, or occurred only in one of the clones: for example, a *P. micans* which was strikingly similar to *P. hentscheli*, with spear-like apical tooth. *P. gibbosum* was also present in clone *b*. A similar aberrant occurred also in Braarud and Rossavik's cultures.

It is interesting from the taxonomic and geographical point of view that aberrants from cultures resembling *P. adriaticum* Schill., *P. arcuatum* Isel., *P. gibbosum*, and *P. hentscheli*, if found in the sea, would be identified as the above species.

Because at the present moment insufficient evidence has been obtained on *P. micans* species, and the nature of aberrant forms resembling other species, we name them *formas*, reviewed as below: (*a*) *P. micans* forma *typica* from plankton, shows slight variation (Figs. 1–3); (*b*) *P. micans* forma *subtypica* from cultures (Figs. 4–6); (*c*) *P. micans* aberrants, similar to species other than *P. micans* recorded in other distant seas (Figs. 11–22).

Aberrants similar to *Prorocentrum spheroideum* Schiller (56, 57), *P. ovalis* Rampi (53). *P. triangulatum* Martin (42), (Figs. 7–10), were common in all clones.

P. micans forma *delicatula* (Fig. 11).

P. micans without the apical tooth, with smooth wrinkled and bubbled cell membranes (Figs. 12–14).

P. micans deformed in various ways, probably as an effect of culture conditions (Figs. 15, 18, 22).

P. micans (clone *c*) with strong side constrictions (Fig. 19).

P. micans like *P. gibbosum* (clone *c*, Figs, 20, 21).

P. micans with apical tooth spear-like, similar to *P. hentscheli* recorded from the Atlantic (Schiller (56, 57)).

I conclude that there is parallelism in morphological variation in *Prorocentrum* cultures as between Braarud and Rossavik's and my observations.

The aberrants of *P. micans* previously described by Bergh (8), Bremen (11), and Hope (after Braarud) are very rare in plankton and their nature is still obscure. It is my view that aberrants in cultures originate as a result of senescence of crowded populations, and are probably affected by the nature of the medium. It may be possible to determine the nature of the various morphological aberrants by isolating them and growing them in clonal populations. However, in later sections it is shown that some morphological aberrants can develop from different forms of cysts, or during different phases of the life cycle.

Size Variation

The *Prorocentrum* species studied vary greatly in length and breadth, both in plankton and in culture. Three groups of species could be distinguished:

(*a*) small species like *P. cordiformis* sp. nov., Figs. 104–107;

(*b*) medium species like *P. pyrenoideum* sp. nov., Figs. 112–120;

(*c*) large species like *P. micans*.

The size of a given species can indicate its genotypic quality, though it varies also according to the individual and with the various phases of development. In *P. micans* and other species the length of cells varies between 20 and 80 μ. Within these limits intermediate cells occur. Individuals after and before cell division or during cyst formation vary highly. The length

and breadth of the motile cells are closely related to the form and shape of the cysts and their method of division. Spherical cysts, after the intramembranous cleavage, give rise to short broad motile cells, while long cysts produce narrow slim daughter cells, changing their shape in the course of later life, enlarging when cyst formation starts. It would be interesting to know the approximate size variation in relation to the vegetative and reproductive life phases associated with the morphological changes. The size variation of *P. micans* given below should also give information regarding the definite life phase in which the measurement was made; no such information is available because of technical difficulties. Dimensions are: from Lebour (38), length 36 to 48 μ, without apical tooth; from Schiller (56, 57, 58), length 36 to 48 μ; from Braarud and Rossavik (7), length 38 to 51 μ, breadth 17 to 38 μ; from present plankton samples, length 36 to 49 μ, breadth 27 to 39 μ; from present cultures, length 20 to 80 μ, breadth 15 to 35 μ.

The Membrane Pores in *Prorocentrum* Species

The membrane pores exist in many groups of plants. The complex pores in the desmids excrete mucus (Lütkemüller (40)). A diversity of pores in fossil desmids is described by Woloszynska (66). In most of the thecate dinoflagellates various types of membrane pores are found. In Prorocentridae membrane pores constitute typical generic features as demonstrated by Braarud (3) in the genus *Porella*.

In *Prorocentrum* the membrane pores vary in number and pattern according to the species. They change in individual cells according to their age and life phase. The membrane pores usually correspond to the number of trichocysts.

The membrane pores observed in plankton samples are well developed and mostly represented by tubular types of pores. In the cultures the tubular, transverse, and marginal pores almost disappear, though in a few individuals they remain. The distribution of pores in plankton and culture changes (Figs. 1–6). The membrane pores are classified, according to their shape and position within the valves, as:

f.p., flagellar pores, anterior and posterior (Figs. 25–27);
a.t.p., anterior tubular pores or simple pits (Figs. 1–3);
t.t.p., transverse tubular pores usually separated by central poroid field (Fig. 3);
p.ap., posterior tubular pores arranged in arches (Figs, 1, 2, 4, 6), transformed into simple pits in cultures;
p.p., posterior pores, a characteristic design for species with four to eight pores (Figs. 1–6);
m.p., marginal pores (Fig. 3);
s.p., suture pores at upper edges of the suture in *P. micans* and *P. redfieldi*;
t.f.p., tiny pits around flagellar tooth concavity (Fig. 25).

The transverse pores have larger outlets at the membrane surface, but smaller outlets at the surface in contact with the protoplasm. Their canals

are also often twisted. At the outlet of the pores a higher ring of membrane encircles the edge of some pores. The pores of ventral and dorsal valves build up symmetrical or asymmetrical patterns. The space between the pores is covered with punctuation, coarser towards the central poroid field, which consists of a system of small holes at the surface of the membrane but not perforating it. The tubular pores are usually turned towards the long cell axis with the exception of the pits. Thin-membraned individuals, examined after shedding their valves, showed the presence of the membrane pores. A peculiar pattern of membrane pores, consisting of two rows along the wide cell surface edges, occurred as an aberrant form in cultures (Fig. 6).

The short tubules were also observed in cultures of *P. micans* forma *delicatula* (Fig. 11). In the large spherical or subspherical species such as *P. scutellum*, *P. micans*, and *P. levantinoides* it is possible to identify them by a pore formula, though their arrangement varies. In narrow cells the number of pores and their structure is reduced, often to one or two rows of membrane pores. The spherical species like *P. pomoideum* are perforated with pores which bear specific features but are not arranged in a special pattern. This was observed by the author in *P. robustum* Tafall, *P. maximum*, *P. sigmoides* Tafall (45).

The hyaline, structureless valves of young cells stained with cresyl blue may reveal very delicate pores. Strong-membraned cells should therefore be chosen for studying membrane pore features.

The Apical Tooth in *Prorocentrum* Species

The apical tooth, an organ of unknown origin and purpose, represents a principal taxonomic criterion for distinguishing between the genera *Exuviella* and *Prorocentrum*. It may disappear in cultures (Barker (2)); also sometimes it is absent in plankton in *P. scutellum* (Martin (42)). According to the present author the apical tooth disappears in cultures of *P. micans*. In *P. pyrenoideum* and *P. levantinoides*, it could also be partially or completely reduced. Even in complete absence of this organ, the organisms could be identified by other typical membrane features. In *Desmocapsa*, *Porella*, and *Exuviella* small spines anteriorly located, as well as protoplasmic features, distinguish the genera.

Within *Prorocentrum* species the apical tooth is shown to be a highly variable and complex organ, spear-like in *P. hentscheli*, eminent and hollow in *P. dentatum*, sometimes containing protoplasm. Small *Prorocentrum* species have a simple apical tooth, reduced merely to a few spines located around the flagellar pores. The rudimentary apical tooth in *P. cordiformis* consists of two spines (Fig. 31; details concerning apical tooth structure are included in the figure explanations). In *P. pomoideum* sp. nov. the third spine is located between the flagellar pores (Fig. 32). In *P. redfieldi* (Fig. 34) the hyaline membrane stretches between two spines. A single spine and wing occur in *P. pyrenoideum* (Fig. 33). In *P. scutellum* it consists of a strong short spine, wide wing, and two or three small spines (Fig. 35). Well

developed in plankton, its form in *P. micans* (Figs. 36–41) is very complex and changing in details. It is undoubtedly different from *P. levantinoides* sp. nov. (Figs. 42 and 44). The forms and structures of the apical tooth in cultures are more variable than in nature.

Lebour (38) described the division of the apical tooth in *Prorocentrum* after the splitting of an isosceles organ into two even parts. Various forms of this organ were studied in cultures by the author. An individual *Prorocentrum* can develop a prominent apical tooth or it may be absent. Some cells possess only small spines resembling *Exuviella* species. As observed in cells produced after posterior division (Fig. 55), no apical tooth was formed or it was very rare. The apical tooth could be formed also inside the cysts. In cysts which elongate and become dorsoventrally flattened it is formed by exogenous droplets of ectoplasm, in the anterior part of the cell. In the motile cells from which naked protoplast escapes after the valves have opened it springs out immediately as a hyaline apical tooth (Fig. 85).

In spherical and subspherical cysts the apical tooth was formed during the course of their transverse constriction. The delicate stratification in the strong membrane cells was usually present. In rare cases the large apical tooth shows that there is a hollow capillary space which starts from the apical tooth base and ends at the top of the main spine. It is of phylogenetic interest that in clones of *Exuviella* species, grown from single cells, in rare cases the apical tooth occurred as a typical *Prorocentrum* apical tooth. These prove that both genera are nearly related (my observations).

Flagellar Pores and Closing Apparatus of the Valves

Two flagellar pores have been reported in most of the *Prorocentrum* species, and my observations agree. Single flagellar pores were recorded in *P. arcuatum*, *P. ovula*, and *P. nanum* by Schiller (57, 58). Two flagellar pores drawn by Biecheler (10) are of the same size, and are shaped like small boutonnieres as observed by the author. Two pores were observed by the author (Fig. 25); from the larger the longitudinal flagellum emerges, and from the smaller comes the transverse flagellum. The pores are also named "posterior" and "anterior". In aberrant individuals in cultures two pores perforating each valve have occurred (Figs. 26, 27). The flagellar pores are associated with the apical tooth and the complicated system of intravalvar structures (Figs. 36, 44, 46, 47). The diagnostic value of these structures is certain. It is convenient to call the perforated membrane valve by the flagellar pores the ventral valve, and the opposite one the dorsal valve. Dorsoventrality thus could be recognized by the presence of flagellar pores even in aberrant *Exuviella*-like forms without an apical tooth, or in other Prorocentridae which lack this organ.

Details of the closing apparatus of the valves were studied in *Porella* by Braarud (3). Owing to the small size of their structures the largest valves were selected for examination. Three modifications of the valvar closing

apparatus were observed by the present author: (*a*) indentations alternating with projections, interlocking the opposite edges of the valves (Fig. 23); (*b*) serrated edges of the sutures; (*c*) smooth edges of the sutures.

My experiments with hypertonic solutions of sea water show various efficiencies of closing forces against osmotic pressure in various species.

Some Features of the Cell Membrane

The viscous ectoplasmatic layer found under the membrane surface of the flagellated organisms produces various membrane structures by its power of coagulation. The evidence of this was obtained by discharging small ectoplasmatic droplets through the membrane pores of various *Prorocentrum* species. Such ectoplasmatic droplets immediately enlarge into large bubbles with solid membranes (Fig. 81, *e*). It was also recorded by the author in various thecate and naked dinoflagellates. The blue coloration of the ectoplasmatic bubbles of the rigid walls indicates cellulose. The cell membrane of the adult individuals of *Prorocentrum* species seems to be more complicated. Mangin (41) reported that the peridinean membrane is composed mainly of cellulose, pectins, and callose. The chemical composition of the membrane in *Ceratium* species changes, however, as the season advances, according to Pearsall (after Fritsch (24)). According to Braarud and Rossavik (7), the cell membrane of *Prorocentrum* shows cellulose reaction. According to the present writer, the staining capacity of *Prorocentrum* membranes is different in the young and adult phases. The single-layered young active individuals show even absorption of the vital cresyl blue, varying in the adult or aberrant specimens. Two- and three-layered cell membranes were composed of cellulose and an admixture of pectins. The inner membrane usually contained more pectins than the outer, indicating greater inclination for swelling. Secondary lamellation was discovered in many cases. Each *Prorocentrum* species concerned possessed some particular specific features. Three-layered membranes occurred in *P. scutellum* (Figs. 14, 18, 22).

Genus *Prorocentrum* Ehrenberg

Reproduction

Reproduction in Prorocentridae shows two peculiar features which probably represent phylogenetic relationships or merely convergences between dinoflagellates and diatoms. One is the method of longitudinal fission in *Prorocentrum*, in which the older valves are retained and two new inner valves are secreted during the separation of the daughter cells. The other is the intercalary growth of the suture which has a striking resemblance to the similar process in diatoms during the formation of auxospores. Reproduction in *Exuviella* recently studied by the author displays lines of development similar to those in *Prorocentrum*. The previous results and observations by Lebour (38), Schiller (56–58), and Braarud and Rossavik (7) are confirmed in this paper. The modifications in cell division observed are classified as below.

(*a*) Constriction starts at the antapex and is completed at the apex. Two even or uneven cells are formed after division (Figs. 49, 50).

(*b*) Constriction starts along the line of the suture and the final separation of daughter cells takes place in the middle (Braarud and Rossavik (7)).

(*c*) Constriction starts from the apex and is completed at the antapex (Fig. 53).

(*d*) Internal, oblique division of protoplast occurs inside the valves (Fig. 51).

(*e*) Lateral division takes place along the wide cell surface in *P. micans* cultures and in *P. pyrenoideum*.

(*f*) Antapical division (Fig. 55) was observed by the author in cultures; during such division three cells were formed (Fig. 54), sometimes attached to the substrate with mucus. Though it is thought that division of *Oxhyrris* (Hall (31)) follows the general pattern of dinoflagellates, conclusions cannot yet be made.

Cysts

Spherical and subspherical cysts in *Prorocentrum micans* were described by Braarud and Rossavik (7). Both types of cysts occurred only in the old cultures of the present author (Figs. 56, 57), but have never been observed in plankton by the author. Cyst development starts in motile individuals which usually enlarge their margins by the intercalary growth. There is no evidence to show why in some cells spherical cysts are formed while in others the cysts are subspherical and elongated. The pressure exerted by growing cysts opens the valves wide, as drawn by Braarud and Rossavik. According to the present author, two daughter cells may develop inside the cyst. The division of nucleus, pusules, and chromatophores starts almost simultaneously in most cells observed by the present author. Cyst membranes are formed from the inner layers of valves which in their early phases seem to be homogeneous. Some cysts (Fig. 52) have heterogeneous membranes (Fig. 58). Liberation of protoplast from the cysts (Figs. 56, 57) was induced by the author by temperature change. The cyst surface can be sculptured with fine dots or long streaks (Figs. 59 and 60), and can be colorless or brown. Cysts can enlarge in length and width. Thin-membraned cysts can produce two individuals after longitudinal or transverse cell division (Figs. 59, 60). Flagella are shot from the outside. Gradual polar rearrangement of nucleus and pusule position, and formation of apical tooth and membrane pores, are accomplished by tiny drops of ectoplasm leaking out. Some cysts were able to grow and divide (Figs. 60, 61, 62). Aberrant types of division are shown (Figs. 63, 64). In Fig. 63 the uneven cells are spherical in cross section, while in Fig. 64 they are dorsoventrally compressed.

Chromatophores

Holophytic flagellates contain leucoplasts, according to Schütt (after Schiller (56, 57)). Carotin, phylloxanthin, peridinin, and phycocyan have been extracted from holophytic forms. Strain (62) has demonstrated the presence of chlorophyll, diadinoxanthin, dinoxanthin, dinoxanthinin, neodinoxanthin, and peridinin. The principal carotinoid in *P. micans* is hydroxylated peridinin (Graham (29)).

A single chromatophore corresponding to the form of the valve is contained in each half of the cell membrane. It varies, according to species, from dark yellow to dark brown. The concentration of the chromatophore pigment depends on intensity of light and age of specimens. In young cells it is dark; in adult cells it is covered by reserve materials. The pigment of the chromatophore layer is separated from the cell membrane by ectoplasm. The surface of chromatophores kept in darkness shows fine mitochondria, stained with Schweitzer solution. They are rod-shaped and consist of cortex and inner fluid in which finer particles perform Brownian movement (Fig. 65). The above elements could be studied also at the surface of the pusule (Fig. 69). Long and short perforations in chromatophores appear during cell division (Figs. 65, 66) and are associated with formation of the trichocysts. In *P. pyrenoideum* (Fig. 67) trichocysts probably originate within regular protoplasmatic areas, observed during cell division. The fragmentation of chromatophores (Braarud and Rossavik (7), Martin (42)) takes place when large quantities of paramylum are grown in the protoplasm. Chromatophores in cysts were mostly disorganized.

Pyrenoids

The occurrence and structure of pyrenoids in *Prorocentrum* and other dinoflagellates have been little studied. They have been drawn in Desmocontae by Pascher (47), in *Heterocapsa* by Schütt (60), in *Peridinium* by Geitler (27), and in algae by Churda (17). Pyrenoids in the genus *Exuviella* constitute the characteristic feature; according to Thompson (63), in some cells they may disappear. Biecheler (10) studied pyrenoids in *Gymnodinium* and *Peridinium*, and created species based upon the presence of pyrenoids. In *P. micans* they are found in some specimens in cultures grown from a single cell; in others, however, they are absent. Biecheler described a large anterior pyrenoid in a *Prorocentrum* identified as *P. micans*. Such, however, was not noticed by the author in this species, either in plankton or in cultures, although several small pyrenoids, about 4–6 μ and from 8 to 28 in number, were observed embedded in chromatophore pigment or loose in protoplasm (Figs. 71–74). Pyrenoids in *P. micans* consist of spherical stroma containing within their margins tiny rodlets of starch, arranged peripherally around the center. The large pyrenoid located within the central part of the chromatophore was distinguished, in most cells, as a colorless body, but sometimes it was covered by chromatophore pigment in which no starch was evident (Figs. 75–78). Its division into two or four parts was also observed (Figs. 75–78).

Single pyrenoids on *P. cordiformis* sp. nov. and *P. pomoideum* sp. nov. possess large stroma. Their starch rodlets have an orange tint.

According to the present author some individuals of the species contain pyrenoids while others have no pyrenoids. It seems, therefore, risky to base taxonomic diagnoses on the absence or presence of pyrenoids alone.

Vital Colorations

According to Dangeard (19), in *Ceratium* a system of small vacuoles which stain with cresyl blue is dispersed over protoplasm. *Prorocentrum* species absorb vital stains differently probably according to capacity of the particular species and the life phase. As soon as a drop of medium containing *Prorocentrum* was covered, gradual absorption of cresyl blue was observed. It appeared first around the pores, the apical tooth wing, and the spines. The delicate blue shadows soon changed into the intensive deep blue. The thin-membraned cells showed homogeneous absorption of cresyl blue. The adult thick-membraned specimens showed more intensive color in the deeper layers. Very intensive blue occurred in aberrant forms, especially in the specimens with bubbles (Figs. 14, 16, 17, 18). This indicates also different properties of the middle membrane layer. The penetration of cresyl blue solution probably takes place only through the membrane pores and trichocysts, the latter slightly colored in the undischarged state. A few seconds after the pigment penetration began trichocysts were discharged inside or outside the cell (Fig. 84) and often were disintegrated. The vacuome elements absorbed cresyl blue later, showing varying degree of colors (Fig. 79). The strong coloration took place within areas of the disintegrated trichocysts, appearing as long dark tongues (Fig. 79). Threads like protoplasmatic structures attached to the membrane remained unstained, then rapidly stained. Thin-membraned cells were blown up, flagella were rejected, and pusules were pushed deep into the cell (Fig. 80).

The "Central Ring" and Pusules

Beneath the central part of the valves in some *Prorocentrum* species there is a structure named by the author the "central ring". It consists of fine amyloid bodies forming a regular spherical ring. This structure is shown in *P. micans*, *P. scutellum*, *P. levantinoides*, and *P. pyrenoideum* (Figs. 64, 79, 82, 133).

The pusules, still enigmatic organs in dinoflagellates, show complex structures in the pelagic forms. In Prorocentridae they are represented by a single pusule with a fine exit canal opening into the flagellar pore (Fig. 79). This organ is extremely sensitive to salinity–temperature changes. It detaches in the early phase of staining with cresyl brilliant blue (Fig. 80). In some *Prorocentrum* specimens two or three pusules were observed. In one case the exit canal of the pusule was fixed in the membrane pore. Pusules contain viscous fluid, not absorbing cresyl blue or neutral red. The delicate hyaline membrane is flexible and colloidal-like. It is covered by a layer of chromatophore pigment in which mitochondria-like bodies are densely located (Figs. 69, 70). Its presence in the motile cells seems to be associated in some way with flagellar movements which cease immediately after the discharge of the pusule. It occurs in all species of *Prorocentrum* concerned, except *P. redfieldi*, continuing still its movements after discharge of the pusule.

Trichocysts

Various types of trichocysts occur in holozoic and holophytic flagellates (Scherffel (55), Parke (46), Chadefaud (15)). Woloszynska (65) discharged trichocysts in *Gyrodinium fuscum*, Conrad (18) in *Chilodinium*. Bütschli (14) and Chatton (16) discovered "cnidocysts" in *Polykrikos* similar to those known in various Coelenterata. Kofoid and Swezy (37) record rhabdosomes in various genera of naked dinoflagellates. Bursa (13) studied trichocysts in *A. semilunatum*. Biecheler (10) has shown these organelles in dinoflagellates. Dragesco (21, 22) investigated trichocysts with the electron microscope. Trichocysts were recorded in all *Prorocentrum* species studied in this work. Trichocysts in *P. micans* and other species were seen well against the colorless pusule background. After treatment with acid (1.0 *N* HCl) trichocysts shrunk on the surface of protoplasm (Fig. 87). They look like long colorless rods in which alternating brighter and darker segments are clearly visible (Fig. 88). Temperature and salinity changes, as observed by the present author, produce external and internal trichocyst discharge or their disintegration into spherical bodies (Fig. 89). Similar effects could be obtained with various reagents such as Javel water, a boiling or cold solution of sublimate, acids, and cresyl blue. Rapid and complete trichocyst discharge in less than three seconds was produced with Javel water (Fig. 94). The length of the discharged trichocysts was more than 15 times greater than in the undischarged phase. The trichocysts discharged with 5% NaOH agglutinate and swell (Fig. 93). Treated with acid vapors they become stiff and break into many parallel needles (Fig. 90). For the first time axion and cortex in trichocysts were observed with front phase illumination. Schweitzer solution and Lugols stain selectively the axion and the cortex, the axion becoming dark violet or brown, the cortex in the beginning yellow or bright violet (Fig. 92). After a few seconds these heterogeneous structures disappear, and trichocysts become uniformly dark. Some discharged trichocysts turn into small droplets (Fig. 91).

It seems peculiar that fresh plankton samples of *Prorocentrum* often do not discharge trichocysts or only from few membrane pores. The trichocysts discharge was often complete when using the culture samples. The mass-discharged trichocysts formed an opalescent sediment found in the culture flask. This sediment contained, however, amorphous, broken, or swollen trichocysts. Since experiments show that penetration of sea water into the protoplasm takes place through the ends of trichocysts locking the membrane pores, trichocysts presumably act as filters in outward and inward diffusion, while the solid membrane seems to be osmotically indifferent. Trichocysts seem to be of no vital importance for life maintenance, since even specimens with completely discharged trichocysts can regenerate their motility again. Even motile *Prorocentrum* presumably in good metabolic conditions rarely contained complete sets of their trichocysts, while a great percentage of culture population possessed partially discharged trichocysts. The swelling of trichocysts or forming of nails or pegs locking the membrane pores (Fig. 86)

seems to be accidental and probably has no particular physiological meaning. It is finally concluded that trichocysts of the type concerned do not serve defense purposes of Prorocentridae.

Experiments with Hypotonic and Hypertonic Sea Water

The purpose of the experiments was to define the osmotic resistance of *P. micans* against hypotonic and hypertonic solutions of sea water, and other biological responses. The osmotic resistance in various groups of algae varies according to environmental conditions (Höfler (33, 34)). According to Biebl (9), deep-water algae have lower osmotic resistance than shallow-water algae; it is from 0.5 to 1.49. Algae exposed to the air have an osmotic resistance of 0.3 to 2.2 in the intertidal area. The limit for the deep-water Rhodophyceae is between 1.4 and 1.5. Higher concentrations become harmful to the organisms. Normal sea water or water 1 taken from 10 meters depth near Plymouth was used, gradual dilutions being obtained by mixing with ordinary tap water. The hypertonic solutions were obtained in a similar way by using water 2, a double concentration of normal sea water. A drop of culture medium containing living *P. micans* was placed upon the slide, then mixed with a drop of distilled water of the same size and observed immediately under the microscope. The results are given in Table I. The investigated organisms demonstrated great sensitivity and even transfer from the culture flask to the slide produced a short shock which rendered them motionless for about a minute. A second purpose was to determine lethal effects of hypotonic and hypertonic solutions. It was observed by the present author that advanced plasmolysis associated with nuclear displacement and trichocyst disintegration does not kill *Prorocentrum*, which may regenerate its structure and carry out all its activities again.

TABLE I

Experiments with hypertonic and hypotonic sea water

Experiment	Concentration	Results
No. 1, with hypertonic solutions	1.50	All cells dead, complete plasmolysis
	1.40	All cells dead, partial or complete plasmolysis
	1.35	All cells dead, partial plasmolysis
	1.30	After 3 to 5 minutes some cells recovered from the shock, trichocysts discharged, flagella rejected
	1.25	After 1 minute some cells motile again
	1.15	Most cells recovered and motile after short period
	1.10	No shock observed
No. 2, with hypotonic solutions	0.25	Rapid plasmolysis and protoplasm discharge
	0.30	All cells dead, cell membrane burst
	0.50	Mass discharge of trichocysts, all cells dead
	0.60	After 1 to 3 minutes some cells recovered
	0.70	After 23 minutes movements restarted in strong-membraned cells; thin-membraned cells burst
	0.80	No shock observed, all cells motile

No harmful effect is caused by the hypertonic solutions of concentration 1.30 and 1.25. The critical concentration associated with mass trichocyst discharge is 1.30. All lower concentrations produce rather small shock or none at all. In the hypotonic solutions of sea water mass discharge of trichocysts and strong plasmolysis occurred at 0.5 concentration. All cells were motionless or dead. A concentration of 0.6 is one from which some cells can recover after 1 to 3 minutes. In a concentration of 0.7 most cells become motile, though young thin-membraned cells disintegrate. No shock occurred at the concentration of 0.8.

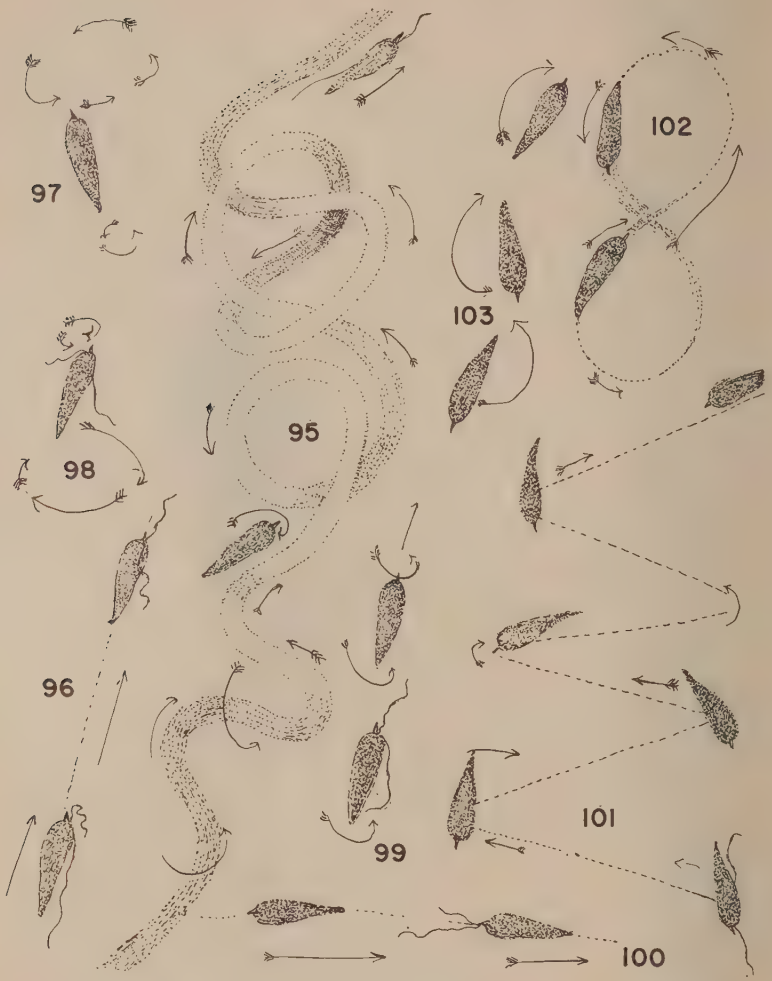
Movements in Prorocentrum Species

Flagellar movements in various genera vary specifically according to Peters (51). *Porella* produces a quick spiralling around an axis which passes through the cilia pole and the center of the cell (Braarud (3)). In *Exuviella compressa* one flagellum is directed forward and the other is whipped about, causing the cell to rotate on its long axis. At the same time the whipping flagellum trails and the cell glides along without revolutions (Thompson (63)). *Haplodinium* and *Prorocentrum* move in similar ways. The thread-like flagellum directed anteriorly pulls the cell forward, whilst the second flagellum swings transversely about causing rotation of the individuals (Fritsch (24)).

The above observations of flagellar movements mostly concern a single short phase of flagellate movement. However, more continuous observations of the movements show unexpected qualities in various species. Eight species of *Prorocentrum* studied by the author produced various types of flagellar movements. It seems almost impossible to describe all the modifications in each organism and the changing positions of the flagella and of the cell. Special attention was taken, therefore, to investigate various positions of the cell during movement. From observations on *Prorocentrum* genus, each species possesses its own type of movement, associated with its own morphodynamic features. Motile cells packed heavily with paramylum or strong-membraned aberrants produce different types of movements. In general, small species like *P. cordiformis* show much faster movements than the large *P. scutellum* or *P. micans*.

P. redfieldi was chosen as most convenient for observation. Eight types of movements occurred in this species, Figs. 97-103 indicating the details. More common kinds of movements are shown (Fig. 95), jet-like forward movements (Fig. 96), rotation of apex and antapex (Figs. 97 and 98), rotation through the short axis in a forward direction (Fig. 99), backward zigzagging (Fig. 100), tumbling (Fig. 103), and formation of the figure "8" when fixed with the posterior flagellum at the substrate. Some *Prorocentrum* possessed three flagella, as also reported by Ohno (44) in *Gymnodinium*.

In jet-like backward jumps (Fig. 100) both flagella were almost motionless. It is supposed therefore that some other kinetic forces act in this peculiar case. The anterior seems to be more active than the posterior flagellum. The anterior flagellum, though its position varies from anterior to lateral,



FIGS. 95–103. Movements of *P. redfieldi*. ×500.
FIG. 95. Common type of movement consists of spiralling, looping, combined with gyrating.
FIG. 96. Forward movements in straight lines.
FIG. 97. Rotation of apex in large circles, and of the antapex in small circles.
FIG. 98. Opposite to movements in Fig. 97.
FIG. 99. Forward movements in straight lines, rotation along short axis.
FIG. 100. Backward, discontinuous jumps in straight lines.
FIG. 101. Sudden zigzagging at various angles.
FIG. 102. A cell described a figure "8" while fixed with its posterior flagellum to the surface of the substrate.
FIG. 103. Forward tumbling.

works mostly in front of the cell and around the apex. It also performs vibrations while stretched forward. Its undulations, moving from the tip of the base, vary greatly from large waves of high amplitude to small waves of low amplitude. The posterior flagellum generally occupies a position beneath the cell and trails behind. It seems less flexible than the anterior flagellum though it acts as a steering mechanism in forward and backward movements. Four motile cells of *P. micans* in division performed well-synchronized flagellar actions. No true observations on the flagellar movements can be made under a low power microscope. It is necessary to change objectives from low power to oil immersion very quickly to catch the unexpected jumping, zigzagging, or tumbling.

Recent investigations reveal high structural differentiation of flagella as shown by the electron microscope (Brown (12), Pitelka (52), Houwink (35), and Vlk (64)). The flagellar apparatus in *Euglena* responds to the stigma stimulated by light intensity and quality. It is concluded that hydrodynamics of flagellates and causes of peculiar movements are still unknown. They are, however, of great efficiency if compared with the small size of these organisms performing diurnal vertical migrations within several meters depth as shown by Hasle (32).

Taxonomy

Prorocentrum **cordiformis** sp. nov. (Figs. 104–107)

Heart-shaped cells, wide at anterior end, narrow at posterior end. Short, simple spine located near the edge of the flagellar pore, an additional smaller spine opposite to it. Spherical and subspherical brown-yellow chromatophores, some of them anastomosed. Two uneven flagella emerge from two distinct flagellar pores. Movement fast, spiralling with frequent tumbling. Cell membrane smooth and very thin, shows no trace of membrane pores, flexible, easily deformed. Three to six trichocysts, rod-shaped, anteriorly located. One or two spherical pyrenoids loose in the protoplasm, occupying a central position in the cell. No suture between the valves was distinguishable.

Dimensions.– Length from 6 to 12 μ; breadth from 5 to 9 μ; height of the apical spine, 1.5 to 2 μ.

Occurrence.—Isolated as *Exuviella* sp. No. 18 by Dr. M. Parke, Plymouth, Knap Buoy, June 1949, at a depth of 4 fathoms.

Affinities.– The organism is undoubtedly distinct from any other known species by its small size as well as by other morphological features. It occupies an intermediate taxonomic position between *Prorocentrum* and *Exuviella*. Since it has two uneven anterior spines it seems to be more closely related to *Prorocentrum*.

Prorocentrum **pomoideum** sp. nov. (Figs. 108–111)

Apple-shaped cells in surface lateral view, often with a posterior constriction (Fig. 111). Flattened dorsoventrally, with a well-marked suture. Cell membrane perforated with numerous small membrane pores. Some individuals are covered with tiny spines; other are smooth. Two flagellar

pores, a larger anterior and a smaller posterior one. Two uneven flagella cause fast spiral movement with frequent rotation alternating with tumbling. Main apical tooth without a wing, located at the edge of anterior flagellar pore. Second spine located within the isthmus between the flagellar pores. The third spine shortest, located at the edge of the flagellar cavity. The flagellar pores are embedded in a small shallow flagellar depression forming a separate membrane plate which is fused with the valve. Single large yellow chromatophores are present in each valve, with two anterior perforations where the pusule is located. The posterior chromatophore perforation is found within the nuclear area. In some cells the chromatophore plate is folded around the nucleus. Spherical paramylum grains are present, often bright orange, especially within the posterior part of the cell. Subspherical nucleus close to antapex.

Four to 11 rod trichocysts are present, not attached to the membrane pores, located mostly within the anterior end of the protoplasm. Pusule spherical with a fine exit canal through the anterior flagellar pore. Trichocysts were discharged with Lugol solution, have a small droplet at the top.

Dimensions.—Length from 9 to 20 μ; breadth from 11 to 16 μ.

Occurrence.—Isolated and cultured by Dr. M. Parke, from Yamar Estuary, June 1950.

Affinities.—It seems to be related to *P. obstusum* Schiller (57, 58) from the Mediterranean, which is a larger species with a single apical tooth.

Prorocentrum **pyrenoideum** sp. nov. (Figs. 112–120)

Cells long, fusiform, dorsoventrally flattened, narrow in sagittal view, wide in side view. Posteriorly pointed, anteriorly rounded, with a short apical tooth 1.5 to 3 μ high, placed near the margin of the ventral valve. The apical tooth has a hyaline wing, with a thin main spine. Sometimes an additional little spine is observed opposite the main spine, fused to the wing membrane (Fig. 34). Two uneven flagellar pores can be observed in front view. In side view they are seen as wider and narrower tubules. Flagellar movements are faster than in *P. micans* (Figs. 79–100). The membrane pores within the sutures are mostly simple pits or narrow tubules. In rare cases tiny posterior membrane pores were also observed. Two transverse strips of membrane interlock within the flagellar cavities of both valves (Fig. 113). In aberrant forms a single large funnel-like pore, resembling the membrane pores in *Porella*, was observed in the middle of the valves.

Chromatophores yellow-green, one in each valve, with pusular perforations. One or two large pyrenoids within the center of each chromatophore, in some cells also laterally located.

Posterio-anterior cell division often observed. Some cells with large borders increased by intercalary growth, often square in sagittal view. Cysts spherical. Paramylum as small rodlets or spherical grains. Nucleus subspherical, posterior. *Exuviella*-like cells often observed.

Dimensions.—Length, according to M. Parke, from 10 to 14 μ; length, according to the author, from 8 to 24 μ; breadth, according to the author, from 5 to 13 μ; height of the apical tooth, 3 μ.

Occurrence.—Isolated by Dr. M. Parke, Plymouth, Knap Buoy, June 1949, at a depth of 4 fathoms.

Affinities.—*P. pyrenoideum* shows some affinities to *P. triestinum* Schiller. It is known to the author from his own observations. *P. pyrenoideum* could be distinguished from *P. triestinum* by its large pyrenoid. The large, almost cubic paramylum grains found in *P. triestinum*, also figured by Schiller (56), are thought to distinguish it from other species. Other features characteristic for *P. pyrenoideum* are the long trichocysts located in various positions in the protoplasm (Fig. 118). After discharge they look like long thin needles with a tiny droplet at the top (Fig. 120). The most outstanding structures of *P. pyrenoideum* are long antapical tubular membrane pores (Fig. 118) found only in this species. *P. pyrenoideum* belongs to the medium-size species. It is clearly distinct from any other *Prorocentrum* species described by its *antapical tubular pores*, as well as by other structures. Its closest relative seems to be *P. triestinum* (Fig. 117).

Prorocentrum **redfieldi** sp. nov. (Figs. 121–124)

Cells long and slender, comma-shaped, oval in cross section, rounded anteriorly with a hyaline apical tooth in which the main spine and hyaline wing membrane are fused together, along with the second smaller spine (Fig. 34). Anterior and posterior membrane pores within the shallow depression at the base of the apical tooth. Two uneven flagella with rather changeable movements (Figs. 97–100). Both valves slightly convex, covered by simple pits scattered over the membrane surface with no definite distribution. In some cells the valves are perforated all around their margins. Short tubular pores were observed in only a few individuals. The general morphological features of some cells do not differ much whether seen in sagittal or ventral view.

A single yellow-green chromatophore plate in each valve. Small paramylum grains mostly in the anterior part of the cell, staining blue with Lugol solution. Two regular spherical trichocysts extending between the chromatophores. Pusule reservoir with a fine exit canal within the flagellar pore.

Dimensions.—Length from 18 to 32 μ; breadth from 6 to 10 μ; height of the apical tooth, 3 to 5 μ.

Occurrence.—Eel Pond plankton and in coastal plankton of Woods Hole, Massachusetts, from July to September, 1952. Mostly found in Tintinnidae, which select it as food. Neritic-brackish.

Affinities.—It seems to be closely related to *P. triestinum* and *P. schilleri*.

Prorocentrum **levantinoides** sp. nov. (Figs. 125–127)

Cell shaped like an almond kernel, broad at the anterior end. Strong valves with a prominent apical tooth (Figs. 42–44, 125), unlike *P. micans*. Membrane pores vary in different individuals (Figs. 125, 126), with well-developed marginal pores. Large poroids present in the center of both valves, or in some cells covering the whole valvar surface (Fig. 47). Two smooth membrane surface areas: near the apical tooth and at the edge of the posterior end of the cell. Two brown-yellow chromatophores, perforated by the

pusule reservoir which leads into a flagellar pore. The rod-shaped trichocysts distributed according to the pattern of the membrane pores. Numerous paramylum grains dispersed within the protoplasm. A large "central ring" in the center of the chromatophore. Nucleus posterior.

Dimensions.—Length from 15 to 60 μ; breadth from 18 to 49 μ; height of the apical tooth from 8 to 12 μ.

Occurrence.—Coast of Israel, Tel-Aviv, Jaffa, from June to December, 1944.

Affinities.—It is closely related to *P. micans*, but distinct from it in respect to the apical tooth, membrane pore pattern, and trichocysts. There is a tendency in some cells toward reduction of the membrane pores.

Prorocentrum scutellum Schröder (Figs. 129–134)

Cells oval, laterally compressed, rounded posteriorly or in some cases pointed. The apical tooth short, consisting of a wide hyaline wing attached to the solid spine. The flagellar cavity shallow, one or two small additional spines found opposite the apical tooth. Woods Hole specimens compared with Martin's (42) descriptions and figures show identical features. The pattern of the membrane pores in *P. scutellum* has its own characteristic organization, varying according to the individual specimens. An asymmetrical distribution of the membrane pores within the valves occurred in most of the cells. In a few cases regular distribution of membrane pores was observed. Schiller's (58) drawings compared with the author's show great similarity between Mediterranean and Atlantic forms. The distinguishing three-layered cell membrane was often found in this species. In some cells the edge of the cell membrane is thin, in others wide. In sagittal view the cell is a lens-shaped structure with a well-developed apical tooth. The shape and distribution of trichocysts are specific for this species. Two of them are longer, and reach the center of the cell close to the "paramylum ring". Pusules larger than in *P. micans*, with a fine exit canal leading to a flagellar pore. Movements slow. A large nucleus close to the center of the cell changes to a central anterior or central posterior position. After shedding the flagella, under unfavorable conditions, the protoplasm shrinks, its innermost membrane swells, and it turns into a resting phase which resembles palmella.

Dimensions.—Length from 36 to 57 μ (Lebour (38)).

Occurrence.—It has been reported from the Adriatic Sea, the Bosphorus, the Mediterranean, the English Channel, the Ligurian Sea, the North Sea, and on both sides of the North Atlantic. It seems to be a cosmopolitan species. *P. robustum* Tafall (45) shows some close similarities in the morphology of its outlines. These similarities concern to some degree the structure of the apical tooth. It is certain, however, that the general patterns of the membrane pores in *P. scutellum* and *P. robustum* are different. It should be taken into account, however, that structural details in the former species were not studied. The chromatophore form in *P. scutellum* shows entirely different features from those found in the other *Prorocentrum* species mentioned in this paper. They have many perforations perpendicular to the

edges of the cell membrane occupied by trichocysts (Fig. 133). Some of the perforations divide the chromatophore plate into single lobes, others into secondary snd tertiary lobes.

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Explanation of Figs. 1–94, 104–134

Fig. 1. *Prorocentrum micans* f. *typica*, laid on the dorsal valve, ×1200, from Plymouth plankton. *a.t.p.*, anterior tubular pores; *t.t.p.*, transversal tubular pores; *a.p.*, arch pores; *p.p.*, posterior pores.

Fig. 2. Ventral valve from interior. ×1200. *s.p.*, simple pits; *p.f.*, poroid field; *p.p.*, posterior pores.

Fig. 3. Dorsal surface of the valve. ×1200. *m.p.*, marginal pores; *p.f.* poroid field.

Figs. 4–6. *P. micans* f. *subtypica*. ×1200. Clones *a*, *b*, *c*; *s.p.*, simple pits; *f.p.*, flagellar pores.

Figs. 7–10. Aberrants from the clones *a*,*b*,*c*, (×900) resembling *P. ovalis* Rampi (53), *P. spheroideum* Schill. (59), *P. triangulatum* Martin (42).

Fig. 11. *P. micans* f. *delicatula*. Dorsal valve with tubular pores. ×900.

Fig. 12. *P. micans* (*Exuviella*-like) with smooth membrane. ×900.

Fig. 13. *P. micans*. Wrinkled membrane, from inside. ×900.

Fig. 14. *P. micans*, bubbled membrane. ×900.

Fig. 15. *P. micans*, strong-membraned forms. ×900.

Fig. 16. *P. micans*. Aberrant with ingrowth. ×900.

Fig. 17. *P. micans*. Aberrant with outgrowth containing protoplasm. ×900.

Fig. 18. *P. micans*. Aberrant in which three membrane layers are distinguished after vital staining. ×900.

Figs. 19–21. *P. micans* f. *gibbosum*, in various positions. ×900.

Fig. 22. *P. micans*. Aberrant form. ×900.

Fig. 23. *P. micans*. Closing mechanism of the suture covered with interlocking indentations (*i.i.*). Empty valves in side view. ×1500.

Fig. 24. *P. micans*. Empty valves in side view showing intercalary growth of the borders. *r.*, transverse membrane ribs. ×1200.

Fig. 25. *P. micans*. Front view of the apical tooth. *a.f.p.*, anterior flagellar pore; *t.f.p.*, tiny pits surrounding the apical tooth; *f.a.t.p.*, forked apical tooth part; *a.t.*, apical tooth. ×1200.

Fig. 26. *Exuviella*-like cells of *P. micans* shows flagellar pores, *f.p.* ×1200.

Fig. 27. *Exuviella*-like cell in side view with elongated flagellar pores. ×1200.

Fig. 28. *P. micans*. Observed from inside showing two-layered valve. ×1200.

Fig. 29. *P. micans*. Second inner membrane detached and forming cyst. ×1200.

Fig. 30. *P. scutellum*. Three-layered membrane observed from inside. ×1200.

Fig. 31. *P. cordiformis*. *a.t.*, apical tooth; *s.s.*, small spine. ×2500.

Fig. 32. *P. pomoideum*. *a.t.*, apical tooth; *i.s.*, isthmus spine; *s.s.*, small spine. ×2500.

Fig. 33. *P. pyrenoideum*. Parts of the apical tooth. *w.*, wing; *a.t.s.*, apical tooth spine. ×2500.

Fig. 34. *P. redfieldi*. *a.t.s.*, apical tooth spine; *w.*, wings; *s.s.*, small spine. ×2500.

Fig. 35. *P. scutellum*. *a.t.s.*, apical tooth spine; *w.*, wing. ×2500.

Fig. 36. *P. micans*. Laid upon ventral valve. *a.t.s.*, apical tooth spine; *w.*, wing; *i.s.*, isthmus spine; *b.s.*, two base spines; *a.f.p.*, anterior flagellar pore; *p.f.p.*, posterior flagellar pore; *e.s.*, edge spine. ×2500.

Fig. 37. *P. micans*. Focussed in middle depth. *t.l.w.s.*, transverse low wing support; *f.c.*, flagellar cavity; *a.t.s.*, apical tooth spine. ×2500.

Fig. 38. *P. micans*. *a.t.s.*, apical tooth spine; *u.w.s.*, upper wing support; *f.c.*, flagellar cavity; *e.s.*, edge spine; focussed at the bottom. ×2500.

Fig. 39. *P. micans*. Upon the dorsal valve is shown the deep edge of the flagellar cavity formed by the extended apical tooth spine. *u.w.s.*, upper wing support; *e.s.*, edge spines. ×2500.

FIG. 40. *P. micans.* *l.w.s.*, lower wing support; *c.*, five deep carvings at the bottom spine, stratification of the wing membrane; *a.f.p.* and *p.f.p.*, anterior and posterior flagellar pores. ×2500.

FIG. 41. *P. micans.* Ventral valve after removal of the apical tooth, showing the interior; *t.s.*, transverse membrane strips, a part of the locking apparatus. ×2500.

FIG. 42. *P. levantinoides.* *a.t.*, apical tooth spine; *w.*, wing; *f.c.*, flagellar cavity, intermediate position between lateral and sagittal surfaces. ×2500.

FIG. 43. Position reversed from Fig. 42. *e.s.*, edge spines. ×2500.

FIG. 44. *P. levantinoides.* Sagittal view. ×2500.

FIG. 45. *P. levantinoides.* Apical tooth and flagellar cavity, surrounded by a ring of simple pits. ×2500.

FIG. 46. *P. levantinoides.* Apical tooth from front view, and the structure of the membrane in the flagellar cavity. ×2500.

FIG. 47. *P. levantinoides.* Interior of the valve showing apical spine and wing, and the structure of the flagellar cavity. Dotted points poroids. ×2500.

FIG. 48. *P. micans.* Squeezed from the small sponge found in the plankton sample from Plymouth, England. ×250.

FIG. 49. *P. micans.* Early peripherical constrictions in cell division. ×1200.

FIG. 50. *P. micans.* Advanced antapical division. ×1200.

FIG. 51. *P. micans.* Oblique division of the protoplast. ×1000.

FIG. 52. *P. micans.* Individual in which the inner membrane layer is detached from the outer. ×1200.

FIG. 53. *P. micans.* Antapical cell division in its initial phase. ×1200.

FIG. 54. *P. micans.* Agglutinated three daughter cells in antapical division. ×1200.

FIG. 55. *Exuviella*-like cells after completed division. ×1200.

FIG. 56. *P. micans.* Excystment of protoplast produced by transfer from refrigerator to room temperature. *n.*, nucleus; *v.*, vacuole; *ch.*, chromatophore. ×1200.

FIG. 57. *P. micans.* Two daughter cells in the subspherical cysts. *p.*, pusule; *n.*, nucleus. ×1200.

FIG. 58. *P. micans.* Transverse constriction in a cyst with two-layered membrane. ×1200.

FIG. 59. *P. micans.* Initial phase of transverse cyst division. Brown sculptured cyst surface. ×1200.

FIG. 60. *P. micans.* Motile cyst-like cells; advanced division. ×1200.

FIG. 61. *P. micans.* Two daughter cells derived from spherical cyst. *n.*, nucleus. ×1200.

FIG. 62. *P. micans.* Advanced cyst division in front view. ×1200.

FIG. 63. *P. micans.* Aberrant cells before separation. ×1200.

FIG. 64. *P. micans.* Final phase of cell division shows: *p.*, pusules; *a.t.*, apical tooth formation after division. ×1200.

FIG. 65. *P. micans.* Perforated chromatophore in ventral view. ×1200.

FIG. 66. *P. micans.* Chromatophores segmented in sagittal view. ×1200.

FIG. 67. *P. pyrenoideum.* Regular protoplasmatic territories of chromatophore surface in dividing specimens. ×1200.

FIG. 68. *P. pyrenoideum.* Two chromatophores of a single cell in side view; notice the pyrenoids. ×1200.

FIG. 69. *P. pyrenoideum.* Part of the pusule and chondriosome-like bodies. Chromatophore in the background. ×2500.

FIG. 70. *P. pyrenoideum.* Surface of chromatophore with a mitochondria from which paramylum initiates. ×2500.

FIGS. 71–74. *P. micans.* Various forms of pyrenoids found in unialgal cultures. ×2500.

FIGS. 75–78. *P. purenoideum.* Division of pyrenoids. ×2500.

FIG. 79. *P. micans.* Side view; initial phase of cresyl blue coloration. *v.*, vacuome; *p.*, pusule; *d.t.*, disintegrated trichocysts; *p.g.*, paramylum grains; *p.r.*, paramylum ring; *n.*, nucleus. ×1200.

FIG. 80. *P. micans.* Stained with cresyl blue in sagittal view. *b.e.*, bubbles of ectoplasm discharged through the membrane pores; *m.i.*, bubbles of ectoplasm containing metachromatic elements. ×1200.

FIG. 81. *P. micans.* Partial plasmoptysis produced with hypertonic NaCl 1.30; *e.b.*, ectoplasmatic bubbles (posterior), containing trichocysts, chromatophore pigment, or empty; *t.*, containing only trichocysts. ×1200.

FIG. 82. *P. micans.* Radial arrangement of trichocysts around the "central ring"; *d.t.*, disintegrated rod trichocyst (before experiment). ×1200.

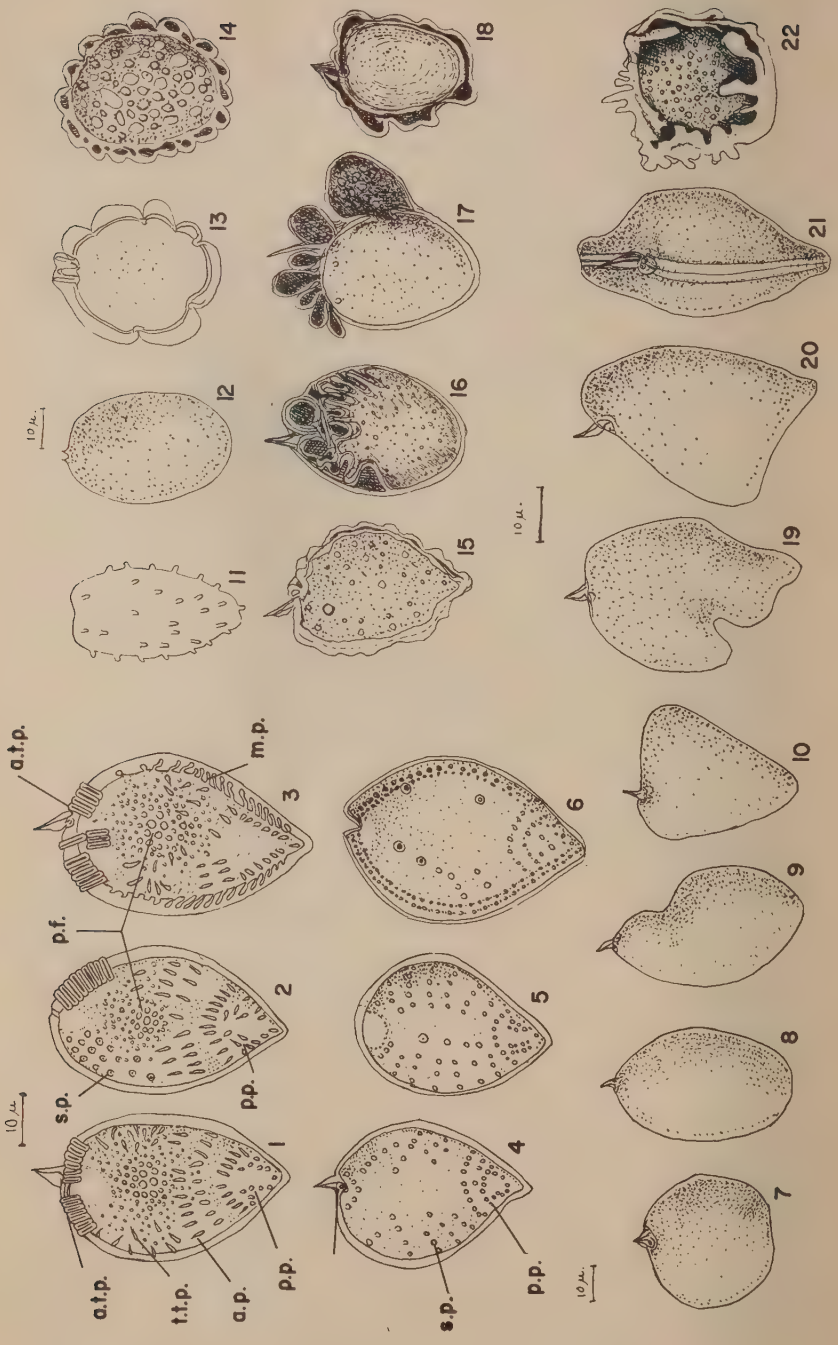
FIG. 83. *P. micans.* (Figs. 82–84, same specimen after a few seconds); after being treated with NaCl solution, trichocysts remained unchanged while protoplasm shrank. ×1200.

FIG. 84. *P. micans.* Destruction of trichoycsts, *d.t.*, into spherical bodies. ×1200.

FIG. 85. *P. micans.* Motile cell after rejection of both valves. ×500.
FIG. 86. *P. micans.* Plasmolyzed protoplasm, pegged membrane pores, with swollen trichocyst, *n.t.* ×1200.
FIG. 87. *P. micans.* Surface location of trichocysts is demonstrated by rapid shrinkage of protoplasm after treatment with acetic acid. ×1200.
FIG. 88. *P. micans.* Trichocysts' structure as observed in vivo. ×2500.
FIG. 89. *P. micans.* Trichocysts after disintegration, stained with Lugol solution and showing bright cortex and dark center. ×2500.
FIG. 90. *P. micans.* Discharged trichocysts, show dark axion and bright cortex, split lengthwise. ×2500.
FIG. 91. Discharged trichocyst turned into fluid droplets. ×2500.
FIG. 92. After treatment with Lugol solution the axion shrunk and broke into dark fragments, while sheath remained unaffected. ×2500.
FIG. 93. Swollen trichocysts after treatment with NaOH. ×2500.
FIG. 94. *P. micans.* Complete discharge of trichocysts, with Javel water. ×1200.

FIGS. 104–107. *P. cordiformis.* Four different individuals, showing pyrenoids, pusules, trichocysts, and chromatophores. ×1200.
FIGS. 108–111. *P. pomoideum.* Fig. 109 in sagittal view; others in ventral view. Trichocysts, membrane pores, pusules, and nuclei. ×1200.
FIG. 112. *P. pyrenoideum.* In sagittal view. ×1200.
FIG. 113. *P. pyrenoideum.* An empty ventral valve seen from inside. ×1200.
FIG. 114. *P. pyrenoideum.* *Exuviella*-like specimen in sagittal view. ×1200.
FIG. 115. *P. pyrenoideum.* Megacyst in sagittal view. ×1200.
FIG. 116. *P. pyrenoideum.* Cyst. ×1200.
FIG. 117. *P. triestinum.* Containing great characteristic paramylum grains. ×1200.
FIG. 118. *P. pyrenoideum.* Typical posterior tubular pores. ×2500.
FIG. 119. *P. pyrenoideum.* Trichocysts' location and pyrenoid. ×1200.
FIG. 120. *P. pyrenoideum.* Trichocysts' discharge. ×1200.
FIG. 121. *P. redfieldi.* Observed in vivo. ×1200.
FIG. 122. *P. redfieldi.* Empty valves. ×2500.
FIG. 123. *P. redfieldi.* In sagittal view. ×2500.
FIG. 124. *P. redfieldi.* Ventral valve, its marginal and posterior membrane pores. ×2500.
FIG. 125. *P. levantinoides.* Laid on the ventral valve. ×1200.
FIG. 106. *P. levantinoides.* An empty dorsal valve. ×1200.
FIG. 127. *P. levantinoides.* A small individual. ×1200.
FIG. 128. *Prorocentrum* sp. with undulated membrane surface. ×1200.
FIG. 129. *P. scutellum.* Ventral valve and its structure (alive), from Woods Hole. ×1200.
FIG. 130. *P. scutellum.* An empty dorsal valve. ×1200.
FIG. 131. *P. scutellum.* Ventral valve shows its membrane pores. ×1200.
FIG. 132. *P. scutellum.* In sagittal view. ×1200.
FIG. 133. *P. scutellum.* Chromatophore structure. ×1200.
FIG. 134. *P. scutellum.* Distribution of trichocysts, observed in vivo. ×1200.

NOTE: Figs 1–94, 104–134 follow.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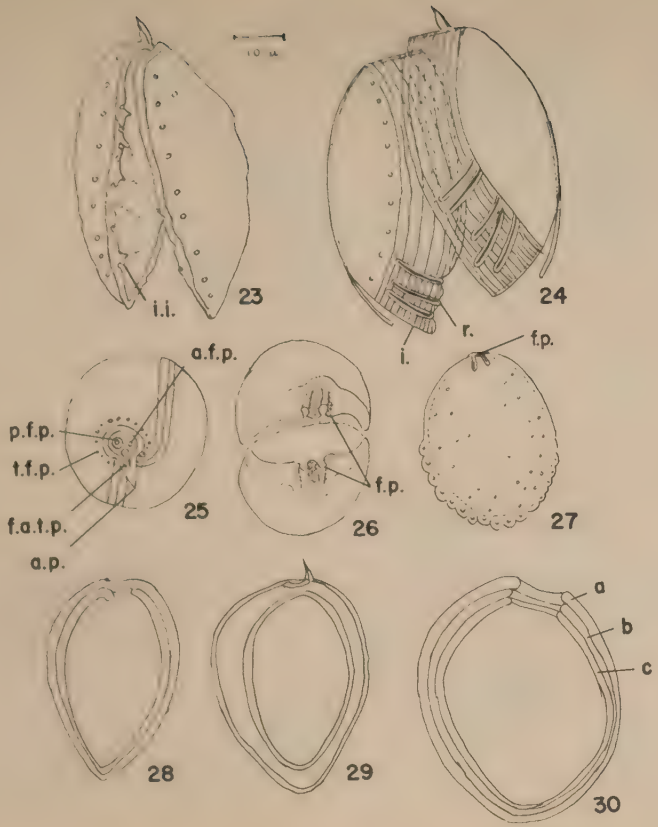
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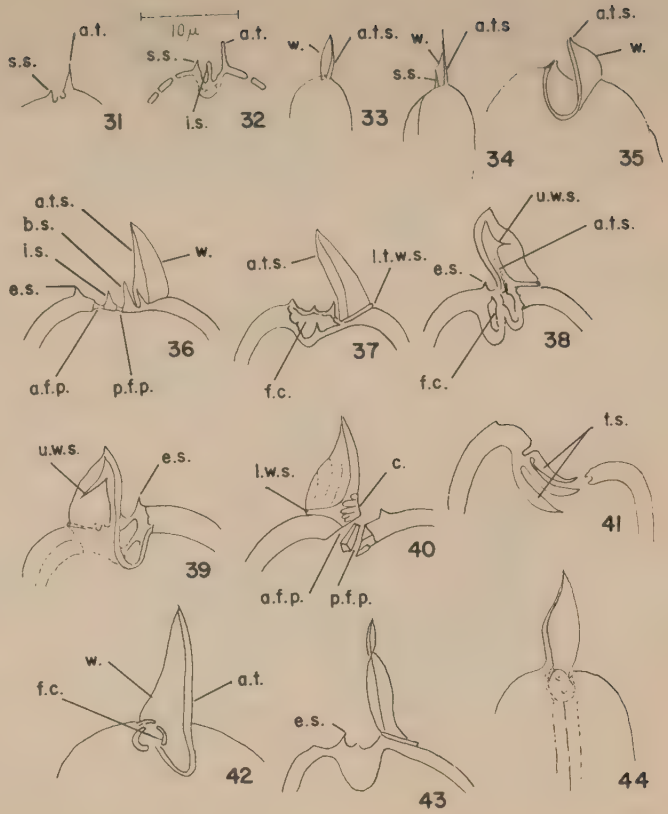
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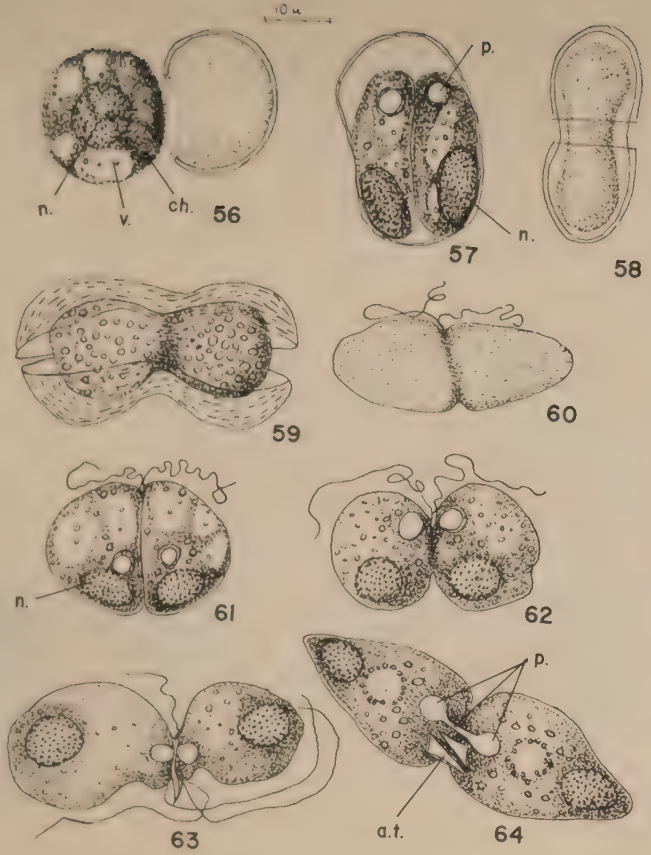
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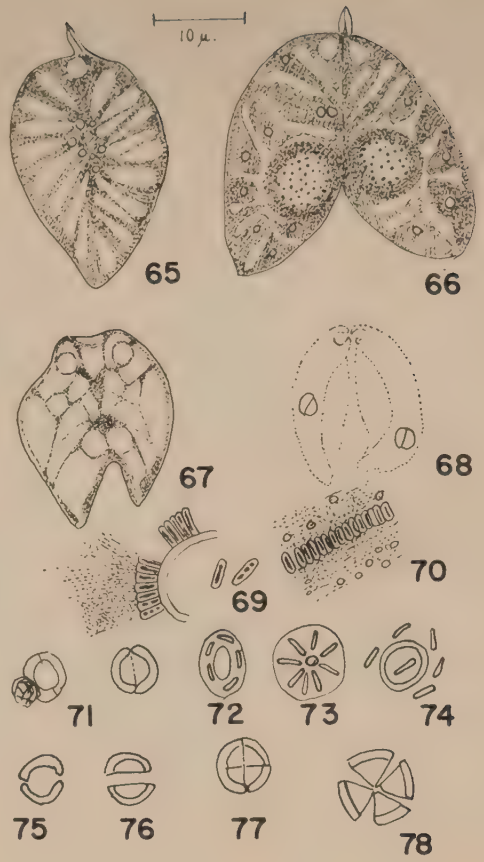
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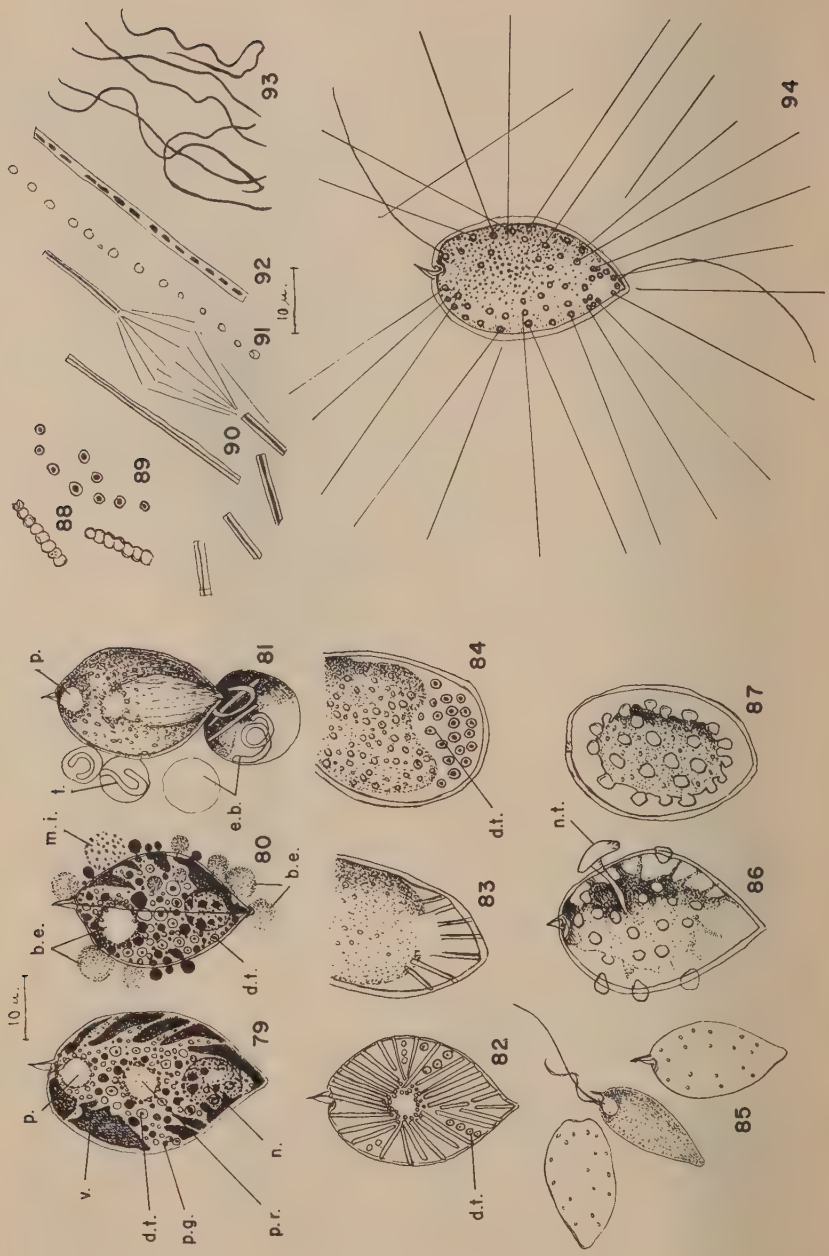
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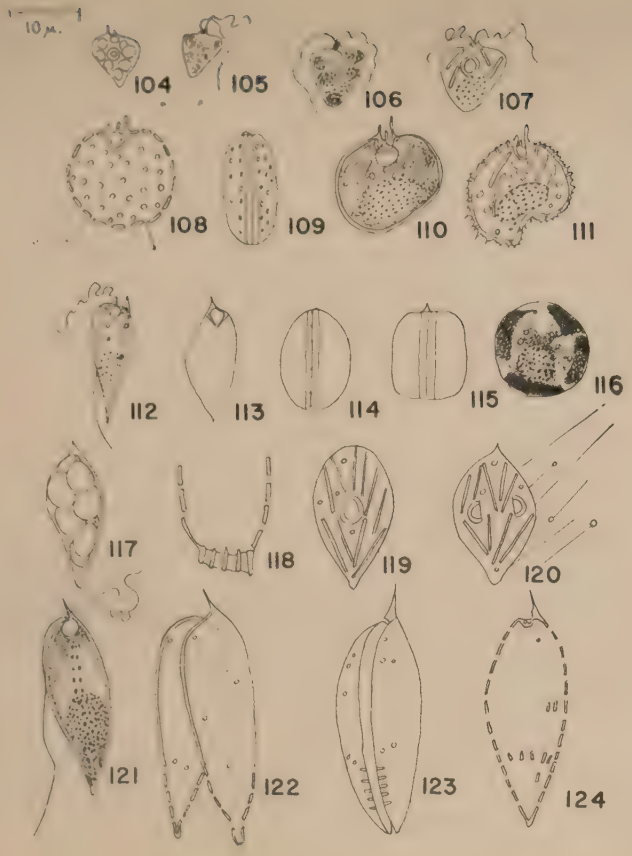
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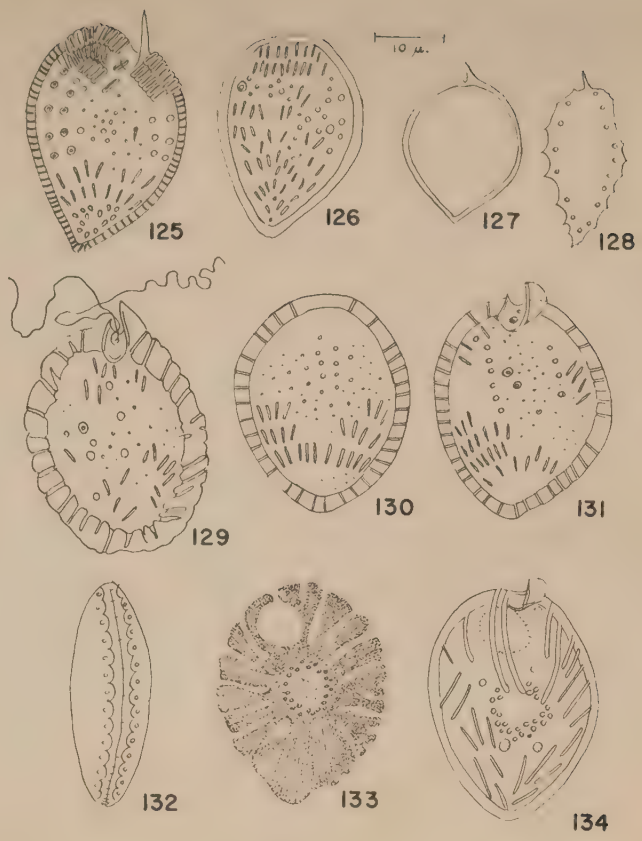
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The Freshwater Dinoflagellate *Woloszynskia limnetica* n. sp. Membrane and Protoplasmic Structures

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SYNOPSIS. A new species of a freshwater reticulate dinoflagellate, *Woloszynskia limnetica,* is described. The membrane structure and the shape of the membrane plates change during cell development. The rod-shaped mitochondria found among the membrane plates disappear in the adult cells; they constitute a center from which starch grains develop. Mitochondria also occur free in the endoplasm. Different physiological activities appear to be performed by morphologically identical chondriosomes. The stigma changes in different phases of the life cycle. The physicochemical properties of the ectoplasm govern the formation of specific structures of the membrane plates, which in turn determine the taxonomic position.

THE ORGANISM used for this study was given to the author by Dr. L. Provasoli for identification. t has been referred to as "*Peridinium* sp.," and was solated in bacteria-free culture by Provasoli from a acterized culture kindly supplied by Dr. E. G. Pringheim. These flourishing cultures allowed serial obervations of the various phases of the life cycle. The rganism is in fact a new species of the rare reticulate enus *Woloszynskia*.

The genus *Gymnodinium* was erected to hold the on-thecate or naked dinoflagellates. According to chiller(22,23) and some recent papers, the genus now ontains 83 species from marine and freshwater habiats. Of these, seven freshwater species were found to ossess a theca composed of small, usually hexagonal lates. The discovery and our entire knowledge of hese thecate forms is the work of Woloszynska(25, 6). Since this primitive kind of theca is delicate, omewhat plastic, and difficult to demonstrate, the genral reaction to her discovery has been to suspect that ost species of *Gymnodinium* will be found to possess ne.

Kofoid(16) states, however, that he has never seen multiplate theca on any of the marine species of *Gymnodinium* (Thompson(24)). Fine membrane eticulation in the marine *Gymnodinium* and related enera was demonstrated by Biecheler(1). Further ontributions were made by Eddy(10) and Harris (14) on freshwater forms.

METHODS

Most observations on *W. limnetica* were made *in vivo*. Phase-contrast microscopy was of great advantage for distinguishing delicate sutures, membrane lates, and mitochondria. Solutions of 0.3% and 0.5% brilliant cresyl blue and neutral red gave good results for vacuome and reserve materials. A droplet of staining solution, dried on the cover glass used to cover the culture medium containing motile *Woloszynskia*, gradually stained them without deleterious effect. The very thin reticulate structure of the membrane was demonstrated with weak and strong Lugol solutions. Rapid preservation and discharge of protoplasm was produced by a boiling 10% solution of mercuric chloride.

DIAGNOSIS

Woloszynskia limnetica n. sp.

Cells subspherical with conical or dome-shaped epicone varying in height. The hypocone at times slightly longer—up to one-third—than the epicone. Epicone a tapered point fusing with the shallow sulcus (Figs. 1, 2, 4). Girdle straight in some cells, displaced about four widths. Left part of the girdle fused with the edge of the hypocone in a joint almost level on the ventral side. Hypocone is broad-ended. One or both lateral edges of the epicone overlap hypocone. Sulcus shallow in epicone, deeper in hypocone. Sometimes the sulcus is noticeable as a narrow strip extending into dorsal area through apex (Figs. 3, 5). It consists of an elongated double row of rectangular, pentagonal, hexagonal or quadrilateral plates. In some cells the sulcus is not found in the epicone. Girdle plates in young cells mostly hexagonal. The sulcus of the hypocone in young cells consists of minute rectangular plates among which metachrome granules are fixed, producing the red appearance of the stigma. In adult cells, the larger membrane plates and wider sulcus are built up of two rows of square plates. A finer reticulation as small hexagonal plates occurs in young motile individuals. Flagella, twice or 1½ times as long as the body, produce a fast, spiralling movement along the short axis of the cell combined with gyration. The path of motion is very similar to that observed in vari-

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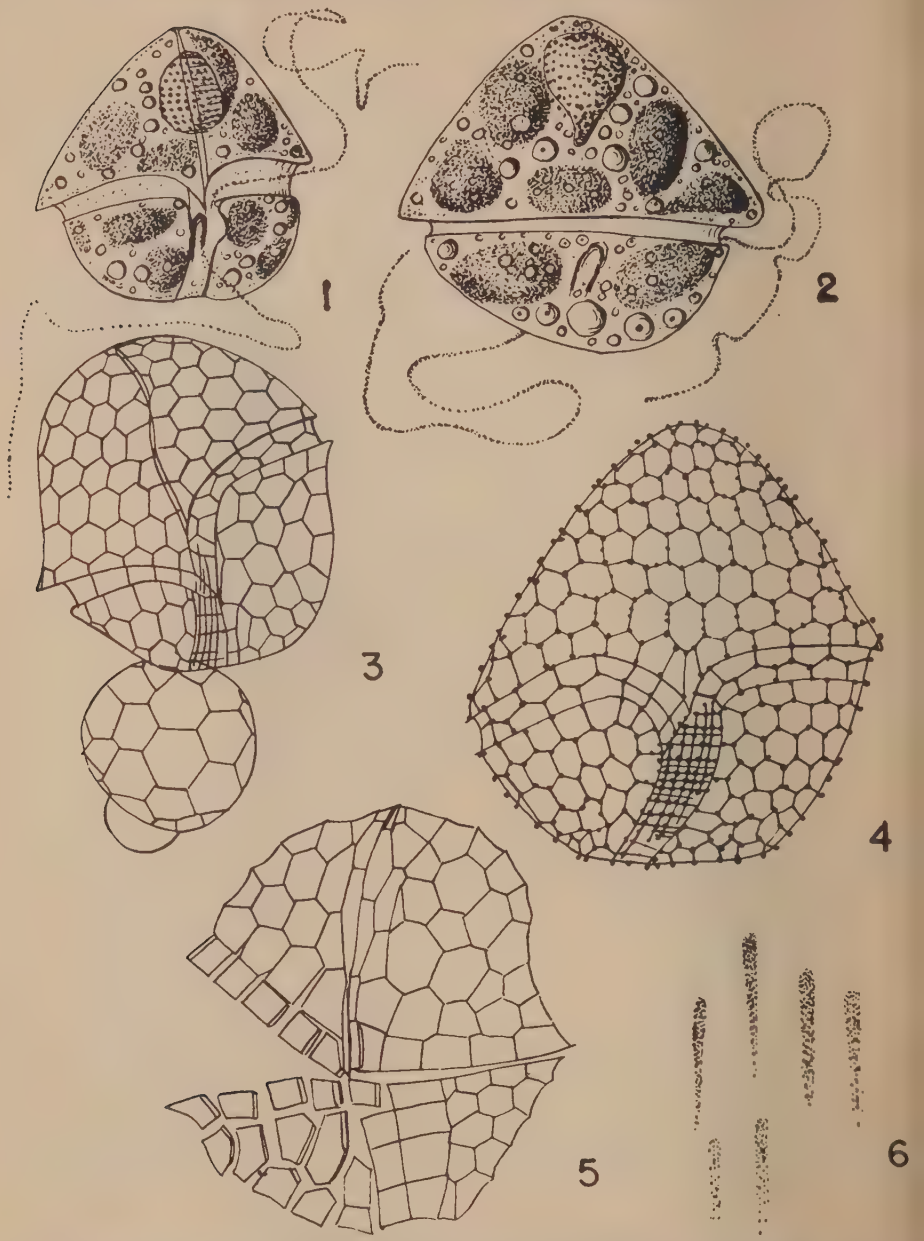


Fig. 1. *W. limnetica* in ventral view. Fig. 2. In dorsal view, chromatophores subspheric, V-shaped stigma, nucleus. F
3. An empty cell membrane with solid ectoplasmatic discharge containing secondary hexagonate structure. Fig. 4. Th
membrane of cell showing regular polygonal reticulation of the theca. Mitochondria fixed within the sutures emerge over t

ous species of *Massartia*. Chromatophores spherical or subspherical, 5 to 9 in each cell; in some cells, however, elongated, like an exclamation mark (Fig. 6) and very numerous. Nucleus apical, pear-shaped or spherical, screened by pigment. Pusule spherical with fine exit-canal close to the centre of epicone. Stigma brown-red, V- or U-shaped or irregular. Protoplasm dense, filled with starch grains. Spherical and subspherical cysts with fine hexagonal membrane reticulation or hyaline.

Cell dimensions: Length 28.43 to 63 μ, breadth 11.48 to 60 μ. Cysts of two kinds, spherical and subspherical, of varying diameter from 35 to 69 μ.

AFFINITIES AND OCCURRENCE

W. limnetica seems to be a close relative of *W. hiemale* Wolosz. nov. comb. Thompson(24) on one side; on the other it shows morphological similarities to *W. tenuissima* Lauterb. nov. comb. Thompson (*G. hiemale* Lauterb.). As far as the general morphological features of both forms are concerned, each represents a distinct species. As *W. tenuissima* Lauterb. was studied *in vivo*(22), it was compared with our species. It shows, however, entirely different protoplasmic features. Comparison with *W. hiemale* brings forth similar results. It is also obvious that *W. limnetica* is well distinguished from any known similar species of its genus which is characterized by cysts ornamented with warts, spines and horns, as described by Woloszynska (25,26), Lindemann(17) and Thompson(24). The exclusion of the genus *Woloszynskia* from the genus *Gymnodinium* seems to be fortunate from the taxonomic point of view. Each of the 10 species recombined by Thompson(24) shows some common morphodynamic features of the entire group. Supposedly further elimination of the morphological sets of forms may allow closer estimation of their phylogenetic affinities. *W. limnetica* was found in a small pond, near Cambridge, England. It was cultured at the Haskins Laboratories, N. Y.

MEMBRANE STRUCTURE

The membrane plates of Peridineae are likely created by ectoplasm acting together with other live elements of protoplasm. The mechanism by which the regular polygonal membrane plates are formed is, however, obscure. Obviously hyaline ectoplasm found close to the inner surface of the cell membrane solidifies into a solid wall if discharged from the living cell of *Prorocentrum micans*(5). Small ectoplasmatic discharges come out through the membrane pores as tiny droplets. They immediately increase its volume forming empty bubbles. Similar experiments were repeated in *Gymnodinium* and *Gyrodinium* species(6). The solid wall of ectoplasmic discharges has shown, however, dark and bright alternating structures, similar to those found in the membrane from which it was discharged. Two kinds of ectoplasmic discharges occurred in *Woloszynskia*: single bubbles, and bubbles containing secondary bubbles which formed a polygonal reticulum by pressure in the contact ridges (Fig. 3). Similarities between morphological appearance of the membrane plates and hexagonal structures of the secondary bubbles were very close. Obviously the specific properties of ectoplasm seem to be a main factor in producing the structures concerned. The conditions under which ectoplasm repeats its regular structures are different in the living organism and in the bubbles. No answer, however, could be given as to how various types of dinoflagellate membrane plates, so important from the taxonomic point of view, are produced.

The following types of cell membranes of *W. limnetica* were observed. Each type of membrane probably corresponds to a particular life phase and its physiological requirement.

a) thin, hyaline, single-layered membrane shed by the motile young individuals (not figured).

b) thin two-contoured membrane, with five types of polygonal membrane plates and intramembranous mitochondria emerging from the sutures (Fig. 4).

c) cell membrane thicker than in b), without mitochondria (Fig. 3).

d) thick cell membrane with mitochondria levelled with the membrane surface (not figured).

e) Thick cell membranes with larger membrane plates, (no mitochondria) (Fig. 5).

f) cysts with many-layered, lamellated membranes without detectable polygonal structure (Fig. 12).

g) cysts with single membrane and fine hexagonal reticulation or many lamellae of identical structure (Fig. 10).

h) spheric and subspheric cysts with no detectable structure but smooth or wrinkled surface (Fig. 11).

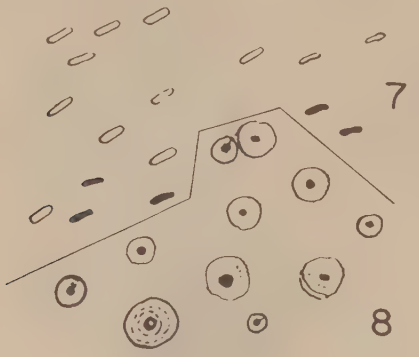
Absence of the mitochondria in thick-membraned cells which correspond to the adult phase, or colorless mitochondria, probably shows a decreasing metabolism in contrast to the young motile individuals with mitochondria emerging over the membrane surface as warts. Thin-mono-layered and polylayered membranes probably represent various modifications in which osmotic activities are regulated by protoplast. No facts are

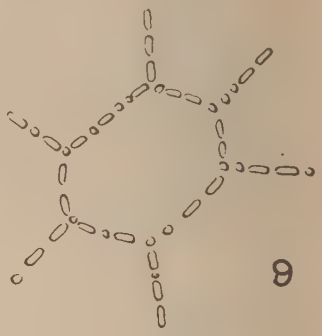
surface of theca. Stigma within hypocone consists of rectangular plates containing in intersections haematochrome granules (Fig. 5). Theca of an adult specimen. Sulcus plates developed by fusion of tiny plates into two rows of large membrane plates. Long polygonal plates of sulcus extend into dorsal area. Fig. 6. Chromatophores elongated in form of an exclamation mark. Camera lucida, oil Immers. × 1200, Figs. 1-5. × 2500, Fig. 6.

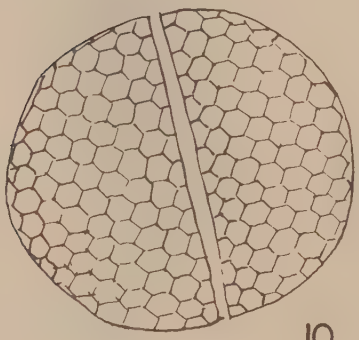
recorded indicating the function of reticulate and hyaline structures in cysts.

MITOCHONDRIA

Chloroplasts and mitochondria are organelles mu







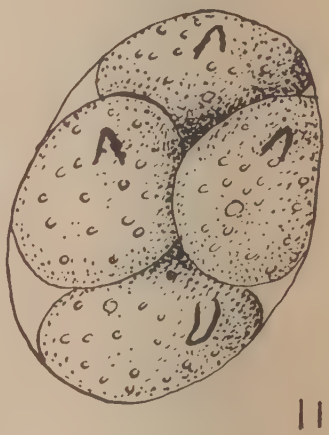


Fig. 7. *W. limnetica* n.sp., mitochondria isolated from the protoplasm and inflated by heat (separated with line from F 8). Fig. 8. Paramylum grains containing red mitochondria. Fig. 9. Single membrane plate with mitochondria inserted wit the suture. Fig. 10. Spherical cyst (broken in the middle) shows smaller reticulation than in motile specimens. No mi chondria observed. Fig. 11. Four daughter cells within elongated cyst. Single hyaline membrane. V-shaped stigmas. F 12. Two daughter cells enclosed within six membranes of the cyst. Camera lucida, oil Immers. × 1200, Figs. 10, 11, × 2500, Figs. 7, 8, 9.

studied under the light microscope(11); the electron microscope has contributed only more details to fit an already outlined frame(27). In *Euglena, Astasia,* and *Distigmopsis* mitochondria are organized in reticula (12.19,21). Similar structures exist in Volvocales(7,9) and filamentous algae(8). Various intramembranous inclusions in marine *Gymnodinium, Gyrodinium* and *Peridinium* species obtained with silver impregnation and *in vivo* as shown by Biecheler(1) supposedly originate from mitochondria. Such inclusions could become specialized in later life phases in different ways(5,6). From the electron micrographs it appears that mitochondria have a limiting membrane and a system of internal ridges that protrude from the inside surface towards the interior(27). Leucoplasts are sometimes indistinguishable from mitochondria, except for their property of synthesizing starch. The plastids may lose their chlorophyll while maintaining their identity and specificity, so far as starch synthesis is concerned (18). The author's unpublished observations on *Exuviella cassubica* Wolosz. show that a reticulated type of chondriom occurs in this genus, but is confined to the young cells. Its continuity is lost when the balance of semi-liquid phase of protoplasm is disturbed by starch accumulation. Rod-shaped, colorless chondrosomes of *Prorocentrum micans*(5) contain liquid, enclosed in the thin membrane. The chondriom of *Woloszynskia* was represented by single rods with a definite membrane and red beetroot-like fluid. *Peridinium balticum* has very similar rod-shaped mitochondria found in the membrane-sutures and within ectoplasm by the present author. The enzymatic activities of the chondriom are currently attracting the attention of many biochemists and physiologists(13,15).

Three types of morphologically identical mitochondria were recorded in *W. limnetica,* differing by their location in the cytoplasm: (a) Surface mitochondria of young active cells fixed perpendicularly to the membrane surface within the sutures of the membrane plates (Figs. 4, 9). It was possible to change their position or move them from the surface by gentle pressing. (b) Mitochondria in the center of paramylum grains. These were isolated from the surrounding protoplasm and inflated by heat until they burst (Figs. 7,8). The discharged red content of mitochondria stained the starch grain reddish, showing the fine concentric rings of its growth. (c) Separate rod-shaped mitochondria also confined to the endoplasm, and located irregularly. The three types of mitochondria probably have different physiological activities.

STIGMA

The stigma in *Amphidinium, Gyrodinium, Gymnodinium,* and *Peridinium* species is usually located in the sulcus area and recognizable by its red-brown color. In various species it is comma-shaped, often reduced to a single red dot. In the genus *Woloszynskia* (17,24,25,26) it is similar to the letter V or U, or hoof-shaped. An irregular net-like form is recorded by Biecheler(1) in *Peridinium foliaceum.* It is generally accepted, especially since the discovery of fine connections between stigma and flagella, that this organelle also acts as a photoreceptor and locomotor center sensitive to various wave lengths(28). The stigma in *W. limnetica* is usually on the left side of the sulcal part of the hypocone, in the form of a U or V, turned down or sometimes up (Figs. 1, 11, 12). It consists of two distinct parts: it is the surface layer of the stigma (Figs. 3, 4) which starts from the membrane surface of the sulcus. In the young specimens of *Woloszynskia* the membrane surface shows fine rectangular or square plate structure with red-brown pigment granules fixed within the corners of sutures. These pigment inclusions are thought to be identical with hematochrom. They are of uneven size. Some of them are smooth, the others have wrinkled surfaces. They are more condensed on both edges of the sulcus. In the adult cells both the membrane reticulation and inclusions of stigma pigment disappear. The tiny plates fuse together and form to rows of large membrane plates (Fig. 5). The described surface structure in young thin-membraned specimens has been found also beneath the membrane surface or slightly deeper. It appears as a homogeneous pigment layer with definite outlines. This structure is considered by the author as a proper stigma. The three dimensional structure of the stigma was confined generally to the thin membraned motile individuals of *Woloszynskia.* In the adult motile forms with well developed strong membranes, only deeper homogeneous layer of the stigma remains. Since in some motile individuals of *Woloszynskia* there is no stigma, it is concluded that this structure is of no vital importance as a photoreceptor. The morphodynamic changes of the stigma which occur in various life phases of the organism indicate that it participates in some physiological activities.

DISCUSSION

The arrangement, size, form and number of the membrane plates of Peridineae served generations of taxonomists as a basis for classification. However, in his comparative studies on the arctic dinoflagellates, Paulsen(20) demonstrates a great variety of morphological forms which he attributes mostly to the lack of sexual reproduction. Many examples of morphological variations found among different thecate dinoflagellates were shown by Braarud(2), Braarud and Rossavik (3) and other authors, who analyzed populations of unialgal cultures. The morphodynamic approach was introduced by Kofoid and Svezy(16) in studying dinoflagellates and was continued later on by Biecheler(1)

both in cultures and plankton. Various dinoflagellates change considerably in cultures even if derived from unialgal populations. They remain, however, "good species" in plankton(3,5). The others show slight or no morphological changes in cultures(2). Comparative studies on unialgal cultures of *Gyrodinium californicum* show that there is a set of morphological forms which is very similar to another set of forms found in *G. pavillardi* Biech. var. *atlanticum*. Both sets of morphological forms differ with only one exception. It is supposed that morphological forms found in *Woloszynskia* represent various life phases or cyclomorphoses(22,23). Since morphogenetic analysis was not applied to establish the taxonomic position of these forms it remains unknown. As suggested by Braarud, subculturing of particular forms may solve some of these problems.

The purpose of the present study was to show morphodynamic changes of *Woloszynskia* as far as cell membrane, chondriom and plastidom are concerned. There was no chance to include nucleo-protoplasmic interactions. The author is convinced that estimations of age phases in motile flagellates could be of definite value. Such estimations have to be very general. It is theoretically possible to define more closely the age of the cell by methods connected with the biology of the particular organism. Estimations should be done preferably *in vivo*. Some attempts to age *Prorocentrum* were suspect, not being based upon long observation of a single isolated specimen(5). As shown in intramembranous chondriosomes, appearance and disappearance of these structures bring forth new facts on the biology of flagellates. The presence of red-blue pigment in chondriosomes of *Woloszynskia* and *Peridinium balticum* lets us make more general conclusions. The appearance of anthocyans in the hypocotyl of *Naias marina* embryos indicates increasing physiological activities and growth(4). Similar increase of activities in young specimens of *Woloszynskia* is associated with appearance of intramembranous chondriosomes containing anthocyan-like pigment. Since these structures are directly exposed in active cells to the water they may participate in osmotic exchange. Disappearance of these structures in adult individuals and cysts shows limitation of supposed functions. Since mitochondria in *Woloszynskia* are morphologically identical and differ by location in the cell, it is proposed to distinguish each type by calling them ectochondriosomes (found in the membrane), amylochondriosomes (found in the starch), and endochondriosomes (found within endoplasm).

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SECTION FIVE

The Origin and Speciation of *Oncorhynchus*

FERRIS NEAVE, F.R.S.C.

THE creation of a new species or a new genus of sexually reproducing animals, through the branching of an evolutionary line, requires that a portion of the original common stock become isolated, so that its interbreeding with the rest of the stock is prevented or greatly restricted. The kinds of barriers which seem to prevent interbreeding between existing species can (for purposes of the present account) be classified as: *geographical*, that is, separation by physical features; *ecological*, that is, separation by differences in habitat; *temporal*, that is, separation by time of breeding; and *generative*, that is, separation by differences in the behaviour associated with reproduction, by nonconformity of generative organs, by incompatibility of gametes, or by differences in chromosomal mechanism.

In considering how isolation is initiated, the advent of geographical barriers undoubtedly offers a satisfactory explanation of many instances of speciation (22; 19). Whether, or with what frequency, other mechanisms play this role has been much discussed. It has been shown, for example, that ecological divergence is often the result rather than the cause of speciation (19). Curiosity concerning the mechanism which originates and maintains separation between species is especially roused by instances in which two or more closely related species are found to occupy the same territory or even the same habitat.

The genus *Oncorhynchus* (Pacific salmons) invites examination in the light of these remarks. It embraces six anadromous species, all closely related. To a remarkable extent these species do not occupy separate territories but coexist throughout an extensive geographical region. The genus itself is very closely allied to *Salmo*, from which it is commonly regarded as being derived (26; 23). Both these genera inhabit the same waters throughout much of the region to which *Oncorhynchus* is indigenous. Since neither the genus *Oncorhynchus* nor its constituent species have travelled far along the paths of evolutionary divergence, the possibilities of their origin can reasonably be sought in geologically recent times.

First, however, the basis for some of the above statements on the relationships and distribution of *Oncorhynchus* can be examined briefly.

Degree of Differentiation Shown by the Genus *Oncorhynchus* and Its Species

Oncorhynchus differs from *Salmo* in that the skeleton of the adult is less ossified. Many small differences exist, for example, in the shape of individual

FRB No. 551

bones of the skull, in the degree of dental development at corresponding stages of the life history, and in skeletal changes associated with the breeding conditions (23). The external character commonly used to diagnose the genus in taxonomic keys—the number of rays in the anal fin—is not an absolute criterion. The two genera are sufficiently similar that the initial diagnoses of acknowledged authorities have not always been sustained, for example, the relegation of the Pacific species of *Salmo* to *Oncorhynchus* by Regan (26) and the description of a freshwater form of *Oncorhynchus* as "*Salmo formosanus*" by Jordan and Oshima (17).

Ecologically the genera have much in common. For example, young *Salmo gairdneri* and *Oncorhynchus kisutch* occur together in many coastal streams, where they share various food supplies (including the fry of *kisutch* and other species of Pacific salmon). Recent investigations have shown that *Salmo* and *Oncorhynchus* also keep company over large ocean areas, where they have many food resources in common (15).

In short, while no taxonomist has questioned recently the validity of the genus *Oncorhynchus*, it is certainly closely allied morphologically to *Salmo* and is not well differentiated from the latter in its ecological characteristics.

Turning from the generic to the specific level, very similar remarks are appropriate regarding the degree of differentiation which is shown by the six anadromous species of *Oncorhynchus*. These species are: *O. masou* Brevoort; *O. kisutch* Walbaum; *O. tshawytscha* Walbaum; *O. nerka* Walbaum; *O. keta* Walbaum; and *O. gorbuscha* Walbaum.

The characters used in practice for distinguishing these species are mainly: the number and relative length of the gill rakers; the number of scales; the number of fin rays; the number of pyloric coeca; and the colour pattern. Although there is some degree of overlapping among species in the numerical range of all the meristic characters commonly employed, it is nearly always possible to identify even single immature specimens with a satisfactory degree of certainty. At the same time, the species are evidently closely related and frequently require careful examination for taxonomic separation. Milne (23), speaking of the species which occur in North America, says: "The Pacific salmon have probably diverged [from a steelhead trout ancestor] into five biologically distinct species in such recent times that they are probably best handled systematically as a species-group." They conform to the definition of Diver (4): "Two or more such species may reasonably be said to be closely related when they are morphologically so similar as to make the allocation of a particular specimen to one species or the other difficult except for an expert; and when there are no grounds for supposing that this close similarity is due to convergence rather than to recent divergence."

The physiological and ecological differences among the species appear to be even less sharp than the morphological distinctions. The preferred temperature for young *Oncorhynchus* in fresh water is 12° C to 14° C for all the species common to Asiatic and American waters. The lethal temperature

was found to vary only from 23.8° to 25.1° (3). In the ocean the preferred temperatures are somewhat lower but again appear to be very similar. Catches of *nerka*, *gorbuscha*, *keta*, and *kisutch* on the high seas of the North Pacific have in each case been associated with temperatures ranging from 5° C to 15° C. Probably *O. masou*, which is confined to the Asiatic side of the Pacific, prefers or tolerates slightly warmer water than the other species, at least when in streams or lakes. However, it occurs with other *Oncorhynchus* in many watersheds and in the ocean is caught in large numbers in company with *O. gorbuscha*. Salinity does not seem to effect any segregation of species within the known ocean range of *Oncorhynchus*. On the other hand, some species (*gorbuscha*, *keta*, and some *tshawytscha*) become physiologically capable of living in strongly saline water at an earlier stage of development than do most individuals of other species (12; 2).

In respect to the selection of spawning grounds, it is well known that certain areas or types of habitat are frequently occupied only or mainly by a single species. *O. kisutch* penetrates the upper portions of many small streams and tributaries; *tshawytscha* typically seeks large streams and lays its eggs in places where the water is relatively deep; *nerka* spawns in the tributaries or outlets of lakes or along the shorelines of the latter; *keta* tends to avoid passage over pronounced waterfalls and hence is often restricted to the lower portions of watercourses; and *gorbuscha* often exhibits a preference for streams of relatively low gradient and fine gravel. Differences in the timing of the spawning period are frequently apparent. Nevertheless, these differences, although sometimes well defined in individual streams, are very far from providing clear-cut general distinctions. Each species at times can be found breeding in company with one or more of the other species.

Similar overlapping is evident in feeding habits. Although average quantitative differences in the kinds of food consumed can be demonstrated, the degree of divergent specialization is not very great. Thus, the "microphagous" *nerka* and *gorbuscha* will also eat fish and squid (6; 15); the "carnivorous" *kisutch* and *tshawytscha* often feed extensively on euphausians (25).

The development of a degree of infertility between diverging stocks (making hybrids less successful than either parental population) has sometimes been thought to be an important factor in the process of speciation. Inability to produce fertile hybrids has been regarded by some as a criterion of the attainment of the status of a species. We shall return to this subject in a later section. However, it may be noted here that at least most of the species of *Oncorhynchus* show a high degree of interfertility. Foerster (11) obtained a high survival of healthy hybrids from most of the reciprocal crossings which he made.

Present Geographical Distribution

One species of *Oncorhynchus* (*O. masou*) is found only on the western side of the North Pacific and, as an anadromous fish, is confined mainly to

the Japanese area and adjacent portions of the Asiatic coast. In a land-locked form it inhabits some of the lakes and streams of Formosa. The other five species occur throughout the North Pacific region, including the Bering Sea. *O. keta* and *O. gorbuscha* are also found sparingly in the Polar Sea, being reported from as far west as the mouth of the Lena and from as far east as the Mackenzie River (30). Except for *masou*, however, the species are sympatric to a remarkable degree, each having its main centres of abundance within the range occupied by all the other species. As already mentioned, common occupancy applies not only to geographic territory but also very frequently to individual streams and spawning areas. On the high seas the species mingle so closely that in drift-net fishing five species are frequently taken in a single "lift."

Present Kinds of Isolation

Although the very considerable extent to which the species occupy the same territories and habitats has been emphasized above, several kinds or degrees of isolation can be observed at the present time.

The celebrated tendency of Pacific salmon to spawn in the streams in which they originated imposes a degree of isolation on the population of every watershed, or even of the separate tributaries of large rivers. Certain statistical differences have been found between many populations. These differences, however, are slight. Some degree of "wandering" is frequently observed, even in the course of observations which extend over a few years only. From the evolutionary viewpoint "homing," in itself, is not a plausible explanation of speciation. It is obvious that the existing species have been able to spread over very extensive areas without undergoing marked evolutionary changes. If, however, geographical changes were to lengthen the distances between breeding populations, homing would undoubtedly increase the effectiveness of such barriers.

At the present time isolation can be seen in some completely freshwater populations of *O. nerka*. In certain instances these populations inhabit lakes which are not visited by anadromous *nerka*. In other cases anadromous fish are present but breed at a different time. Examples of both of these situations are listed by Ricker (27). Similar isolated freshwater populations of *O. masou* occur in Japan and Formosa.

The development of new species of *Oncorhynchus* through isolation in fresh water can be regarded as an evolutionary possibility, although no existing "land-locked" populations in North America are considered to have approached as yet the status of a distinct species. In Japan, several forms of wholly freshwater *Oncorhynchus* have been described as species (16), although the true taxonomic status of these appears to be doubtful. In any case, however, the main trend in the evolution of the dominant existing forms has been towards greater adaptation to marine life (23). This trend has involved the development, at an increasingly early stage of the life history, of an osmotic regulatory mechanism permitting the young fish to

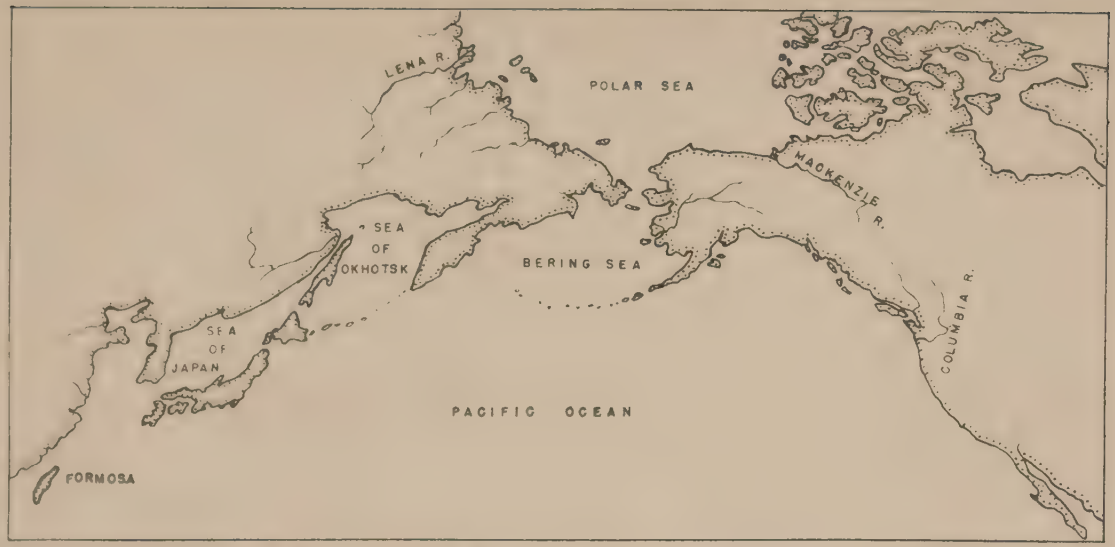

FIGURE 1. North Pacific Ocean and adjacent regions.

tolerate salt water and also the development of a correspondingly early migration to the latter medium. Such evolutionary changes would not result from isolation in fresh water.

A peculiar and very complete form of isolation is exhibited by *O. gorbuscha*. This species invariably, or almost invariably, matures and dies at an age of two years. Therefore fish maturing in "odd" years are segregated in their reproduction from the populations belonging to the "even-year" series. How or when this situation arose is not known. Differences between the two "lines," however, have not hitherto been considered to be of even subspecific value. Presumably the rigidity of the life span and the segregation into even-year and odd-year lines are recent developments in the evolution of *O. gorbuscha*. All the other species of *Oncorhynchus* show variability in the age at which they mature. Some of their males reproduce at the same age as *gorbuscha*. This kind of isolation cannot have been operative in initiating the evolution of the existing species.

In summary, the kinds or degrees of isolation which exist at the present time are not adequate to produce the speciation which has occurred.

Time Periods Required for Evolution

In considering isolating mechanisms which may have existed in former times, it is desirable to form an opinion regarding the period during which such mechanisms can legitimately be sought.

Recent investigators consider that the development of a new species of higher animal is a long-drawn-out process. "Genetic analysis of species-crosses show that in most cases the differences are not such as could have arisen at one step. . . . the occurrence of single mutations can be of little importance in speciation in general. For the significant factors we must look to statistical processes which can carry certain genes to fixation or hold them at certain constant frequencies in the population" (31).

A few taxonomists have implied that new species must have arisen within the period which has elapsed since the most recent Ice Age (about 10,000 to 20,000 years). The many forms of British *Salvelinus* and *Coregonus* which have been accorded specific status, especially by Regan, have presumably been isolated from each other only during a period subsequent to the general glaciation of the region. Djakonov (5) speaks of closely related species of echinoderms occupying the North Atlantic and North Pacific regions respectively. He considers that the separation of the stocks took place not more than some 5,000 years ago. Against these views it may be said that many present-day taxonomists do not regard the aforementioned forms of *Salvelinus* and *Coregonus* as valid species. Regarding the echinoderms, Djakonov himself admits the existence of a much earlier faunal connection (and ensuing disconnection) between the North Atlantic and the North Pacific.

On the other hand, there is a large body of opinion which holds that the evolution of a distinct species requires a far longer period. Ekman (7) says

flatly: "The post-glacial period was, without any doubt, too short to allow the development of new species." Zeuner (32) says: "There appears to be a fastest rate of evolution of species of the animal kingdom under natural conditions, namely about 500,000 years per species-step." Also, "In evolution the number of generations appears to be less significant than the absolute time. . . . No instance is yet known of a species developing at a faster rate than that found in the elephants (about 500,000 years)." Simpson (28) says: "The evolution of a species, fully distinct genetically and morphologically, seems usually to require 50,000 years [*sic*] or more in nature and even in groups with fairly rapid evolutionary rates, such as mammals, some living species are about 1,000,000 years old." According to Mayr (22), "The origin of a new species of higher animals in a human life span is impossible; in fact it may require a minimum of about one-half million years, and normally even considerably more than that."

Origin of the Genus Oncorhynchus

In attempting to apply generalizations such as the foregoing to the particular case of *Oncorhynchus* the following conclusions seem reasonable: (1) Speciation in a group which has shown the plasticity and tendency to local differentiation of *Salmo-Oncorhynchus* may well have been a more rapid process than in many other evolutionary lines. (2) Nevertheless, the processes which resulted in the differentiation of at least six widely ranging species, from a common ancestor, probably began at a time which antedates the present by many scores of thousands of years. (3) The establishment of a genus takes longer than the establishment of a species. The separation of *Salmo* and *Oncorhynchus* must have taken place before the branching of the latter. However, on the generic level, the differences are not great and could presumably have been attained more quickly than those of many other groups which have been accorded the same nomenclatural status.

These considerations, together with the fact that the geographical distribution of the genus is relatively restricted (as compared with *Salmo* and *Salvelinus*), make it entirely reasonable to suppose that all or much of the differentiation and speciation of *Oncorhynchus* has taken place during the Pleistocene epoch. This period has frequently been regarded as comprising about the last 1,000,000 years (18; 10), although some recent estimates, utilizing new dating techniques, have tended to shorten this to about 600,000 years (8).

The view that the spread and diversification of *Oncorhynchus* took place within the Pleistocene receives strong support from another source. At and just before the beginning of this epoch (that is, during the late Pliocene and early Pleistocene), there was "a fairly comprehensive faunistic connection . . . between the cold-temperate faunas of the Atlantic and Pacific" across the present Arctic region (7). Djakonov (5) says: "The palaeontological discoveries confirming the connections of both oceans in the Pliocene are well known and require no further proofs."

The "faunistic connection" permitted many temperate-zone, bottom-dwelling molluscs, some of which are not now found north of the Bering Sea, to spread from the Pacific to the Atlantic. It is hard to believe that under these conditions the genus *Oncorhynchus*, which even at the present time is able to exist along some 3,000 miles of arctic coastline, would not have reached the North Atlantic, by either the eastward or the westward route. If it did reach the Atlantic it is equally difficult to understand why it did not survive there. The physical conditions which have permitted the continued existence of *Salmo* and *Salvelinus* in the North Atlantic region would almost certainly have suited *Oncorhynchus*. The latter has shown itself to be highly successful in cold-temperate streams in competition with these same genera and in the ocean it ranges over great distances. In its occupancy of the epipelagic waters outside as well as within the limits of the continental shelves, *Oncorhynchus* seems to have few enemies or competitors in the latitudes which it frequents mainly.

It may be objected that, even if *Oncorhynchus* did not exist in the North Pacific before Pleistocene times, it should have been able to break out during inter-glacial periods of the Pleistocene. We must admit the near certainty of its presence in the North Pacific before one or more of these interludes. However, in marked contrast with the abundant evidence of Pliocene faunal intercommunication, "very little is known of possible inter-glacial communications between the North Pacific and North Atlantic" (7). It is, of course, well known that communications were entirely severed during parts of the Pleistocene by the Bering land-bridge. It is widely accepted, however, that this barrier was broken during the rises in water level which marked the inter-glacial periods (for example, 30; 20; 21). To this writer the circumscribed distribution of *Oncorhynchus* makes it very reasonable to suppose that throughout the available inter-glacial intervals the physical conditions of the Polar Sea did not become much more favourable than they are at present or, alternatively, that land barriers were more effective. Regarding the former possibility a recent opinion can be cited, namely, that the Arctic Ocean was closed by an ice cover during the inter-glacial periods and that "for the last few thousand years, temperatures have remained about as high as the maximum value that was reached during all Pleistocene inter-glacial stages" (9).

We conclude from the above evidence that *Oncorhynchus* was not present in the Bering Sea region before Pleistocene times. On the other hand, our conceptions at present of the period necessary for the evolution of a new genus (*plus* the amount of speciation shown in the present instance) suggest that the initial separation of the stocks of *Oncorhynchus* and *Salmo* occurred not later than the early Pleistocene. We can therefore look for opportunities for isolation at this period.

The Pacific coastline of North America east and south of the Aleutians, with its narrow continental shelf, has not changed much within the period under discussion. The advent of ice during glacial periods undoubtedly created a broad interruption of the continuity of breeding populations in-

habiting the coasts of the northeast Pacific (*vide infra*). The isolation of southeastern stocks, however, does not seem to have had the effect of converting them into *Oncorhynchus*. On the contrary, anadromous *Salmo* is today relatively more numerous vis-à-vis *Oncorhynchus* in this region than in any other. The many relict or "land-locked" populations of *Salmo* in lakes and headwaters as far south as tropical Mexico point to long-continued occupancy by this genus. *Oncorhynchus* in this sector of its range is not known as a land-locked form and may reasonably be regarded as a more recent arrival.

The Asiatic coastal regions of the North Pacific, on the other hand, are characterized by great gulfs, peninsulas, island chains, and shallow-water areas disposed in such a way that small changes in the relative levels of land and water have greatly changed the position and configuration of the coastline. Physiographical barriers to the spread of faunas and populations have appeared and disappeared. Lindberg (20; 21) has stressed particularly the series of profound changes which have affected the region of the present Sea of Japan. He recognizes three separate periods in which elevation of the land, relative to the sea, resulted in the complete enclosure of a vast shallow-water area. Following each of these periods the sea rose and broke into the area, permitting influx and efflux of populations. Lindberg considers that the first of these periods of land elevation was prior to the Pleistocene and says that in this instance the enclosed water was fresh. The two later recurrences were within the Pleistocene. Of these he says that the earlier resulted in a shallow salt-water sea which may have become brackish at a later stage. He adds that "During this stage and in this body of water, we must suppose, there began the brisk evolution of the endemic marine and brackish water fauna of Japan." According to the same author the cycle of transgression and regression of the sea was repeated at a later period in the Pleistocene. The enclosure of such a great area, situated in an appropriate geographical region and associated with climatic changes, might surely provide opportunity for the isolation and evolution of a stock of salmonids of anadromous or incipient anadromous habits.

It is perhaps highly significant that this is the only coastal area of the world, in this latitude, where *Salmo* is not present today either as an anadromous fish or as relict colonies in fresh water. Since the genus occurs immediately north of the region under discussion (in the Okhotsk-Kamchatka coastal areas) and, of course, elsewhere in the North Pacific, it is hard to believe that *Salmo* never reached Japan or adjacent areas of the Asiatic coast. It can be suggested, rather, that the genus *did* reach the region and that it there became transformed into *Oncorhynchus*. The presence of relict populations of *Oncorhynchus* in the fresh waters of Japan and Formosa, while not necessarily indicative of great geological antiquity, at least points to longer establishment of the genus than in the same latitudes on the North American side where, as already pointed out, the corresponding habitats are occupied by *Salmo*.

It may also be noted that *O. masou*, the species characteristic of the Japa-

nese region today, is most closely related to *Salmo* of all existing species of *Oncorhynchus* (26; while Regan's opinion was presumably based on anatomical similarities, the following ecological or physiological features of *O. masou* can also be regarded as unspecialized or primitive: it is piscivorous; lives both in streams and lakes; is also anadromous, but is not far-ranging in its marine habitat; some males, maturing in fresh water, are said to survive after spawning).

In broad outline, the course of the relationships between *Salmo* and *Oncorhynchus* is envisaged thus: *Salmo* reached the North Pacific via North America. In one of the farthest regions to which it penetrated, a large stock became isolated geographically and diverged markedly from the populations which continued to inhabit other coastal regions of the North Pacific. In due course the newly evolved offshoot spread back through territories occupied by more conservative lines of the ancestral stock. This process of re-invasion was facilitated by increased adaptation to ocean life and was accompanied or followed by a further splitting up into several species.

Speciation of *Oncorhynchus*

The encroachment of the sea on the enclosed waters of the Japanese region presumably coincided with one of the early inter-glacial periods of the Pleistocene and opened up the Pacific Ocean to whatever stock had developed behind the land barrier. It is not necessary to suppose that such a stock would have achieved by that time the status of a new genus, but it might well have established a degree of distinctness sufficient to permit continued divergence when brought into contact with other populations of *Salmo*. Following this presumed broad extension of range, opportunities for further partition of the stock undoubtedly occurred.

Reference has already been made to Lindberg's firm conclusion that the Sea of Japan was again isolated and again drowned at a period some time later than that which he regards as the most potent producer of new evolutionary forms. A further subdivision of the oncorhynchoid stock could therefore have taken place in the area which we have suggested as the most probably starting place of the Pacific salmon offshoot. In view of the primitive character of *O. masou*, however, it seems likely that the development of the more specialized forms took place mainly in other areas. The elevation and submergence of land in the regions now occupied by the Sea of Okhotsk and the Bering Sea paralleled that which has been described for the Sea of Japan. In both of them the presence of wholly or partly enclosed bodies of water at similar time periods has been accepted or suspected (20; 21). These may have provided the isolation necessary for the initiation of one or more lines of speciation. (The possibility that one of them could have provided the actual starting place for *Oncorhynchus* does not appeal to the present writer because of the distribution at present of *Salmo* and *Oncorhynchus*.) Another isolating factor, however, can be suspected of contributing to the development of incipient new species.

During each of the glacial periods an ice sheet, with its centre in British Columbia and southern Alaska, covered a large portion of the North American coastal area. At the maximum development of the ice sheets, all coastal lands from the Columbia River to the base of the Aleutians were completely buried under ice (10). Under such conditions any *Oncorhynchus* stocks which were present on the Oregon-California coast would have been effectively prevented from interbreeding with other populations, even if we assume that such areas as the Bering Sea and Okhotsk Sea were inhabitable.

In general, the periods of land emergence corresponded with periods of heavy glaciation. The ocean regressions which produced the enclosed waters of the Asiatic coast and dried a large part of the Bering Sea presumably coincided with the obliteration of part of the North American coastline by a heavy ice cover. While Lindberg, however, recognizes only two major land emergences and submergences within the Pleistocene epoch, geologists accept the occurrence of four separate major glaciations of fairly equal extent in the northern hemisphere. There seems to be direct evidence of at least three of these in the British Columbia coastal area (1).

Without further evidence, or further analysis, it would be idle to speculate on the sequence of events which has led to each of the existing species. Assuming, however, that *Oncorhynchus* diverged from *Salmo* at or near the beginning of the Pleistocene, the subsequent repeated appearance and disappearance of land and ice barriers in the North Pacific would seem to have created favourable conditions for rapid development of a series of divergent forms and for rapid dispersal of the latter.

Maintenance of Separation

The several periods of geographical isolation which have been postulated are each of the order of 50,000 to 150,000 years' duration. It is not necessary to conclude that species became fully differentiated within these periods. Whether they did or not, however, the diverging populations must have achieved a degree of "separateness" which would enable them to resist merging after geographical barriers were removed.

The process of speciation has frequently been regarded as entailing the development of a degree of sterility between the diverging populations. According to Mayr (22) the following events take place in an isolated population which is in process of evolution into a new species:

> A. Genetic changes owing to mutation, recombination, and random fixation, strongly influenced and directed by selective pressure.
>
> As a consequence of A.
>
> B. (1) The development of reproductive isolating mechanisms
> (2) Ecological divergence
> (3) Sufficient genetic change to make hybrids between parental and incipient species inferior in competition with either parental population.
>
> . . . the development of B(1), B(2) and B(3) must occur while the respective populations are still in the stage of incipient species.

Lack (19) lists "partial inter-sterility and partial ecological divergence" as being associated with the process of speciation.

While it can be accepted that continued evolutionary divergence must at some stage lead to gametic incompatibility, it does not seem at all necessary to assume that sterility (either total or partial) has any special relationship to attaining the status of a species. Moore (24) reported that crosses between northern and southern populations of a single species of frog (*Rana pipiens*) form very defective hybrids, although the same species shows complete interfertility with several other species of *Rana*. Carl L. Hubbs (13), from his experience with many species of freshwater fishes, says: "Degree of fertility is positively correlated with degree of relationship, but only in a rough way." Also, "It is to be expected that bonds of intergradation may be continuously retained by some forms which have undergone an amount of differentiation that would ordinarily accompany complete speciation; or that two forms long separated and well differentiated may retain potential interfertility, so that they will again intergrade and become subspecies when their ranges come to overlap." This last statement, like the previous quotations from Mayr and Lack, seems to imply that when two divergent populations are brought into contact with each other, mating is, or can be, at random. If a high degree of interfertility has persisted, a merging of stocks can take place. If the degree of interfertility has been impaired, natural selection will operate to eliminate hybrids.

Such interactions, while no doubt occurring between some populations, are *not* characteristic of the relationship between the species of *Oncorhynchus*. In this genus, as already pointed out, opportunities for interspecific hybridization are numerous and a high degree of fertility has been shown for various crosses. Even if it be true that the hybrids are at a disadvantage under the action of natural selection, they would certainly be produced in large numbers if random mating were general. For example, many thousands of breeding *gorbuscha* and *keta* are frequently concentrated together as closely as the spawning fish will tolerate. Nevertheless, natural hybrids between any of the species of *Oncorhynchus* are extremely rare. In mixed concentrations of spawning fish pairings take place exclusively, or nearly so, between individuals of the same species. In other words, the fish recognize and breed with their own kind and the degree of interfertility between different kinds does not enter into the matter.

In this connection it is especially to be noted that the various species of *Oncorhynchus* (which during most of their life span resemble each other so closely as to invite frequent confusion) all assume distinctive and conspicuous features of colour, and often of shape, at the time of breeding. This exemplifies Tinbergen's (29) generalization that in closely related species "there is always some striking difference between mating patterns, at least if spatial (geographical or ecological) or temporal (differences in breeding season) separation does not render this unnecessary."

Tinbergen, however, sees the development of distinctive colour patterns

and so forth as a sequel to the loss of a degree of interfertility. "Mating between different species therefore often results in fertilized eggs which are unable to live . . . in less serious cases the hybrids may live but are less vital, or infertile. This premium on intraspecific mating has led to the development of differences between the mating patterns of different species, so that each individual can easily 'recognize' its own species" (29, p. 23).

This view clearly implies that the recognition characters have evolved by natural selection while the two species are in contact with each other. If, however, two closely allied forms are to maintain their separate identities while occupying the same territory, the critical time will be when they first come together, that is, when divergence (morphological, gametic, ecological, or behavioural) is at a minimum and the advantages of separation are also minimal. To begin only at this point to evolve a mechanism to stay separate would be ineffective. Natural selection can be expected to produce changes only in proportion to the survival advantages which such changes confer. The existing evidence indicates that the survival advantages of non-crossing of species of *Oncorhynchus* are small. Yet the mating patterns apparently impose a segregation which is almost absolute.

The opinion presented here is that a mechanism for preventing extensive interbreeding must have been evolved *before* the species (or incipient species) came together. The different mating patterns of very closely allied species inhabiting the same territory are not looked on as an outgrowth of coexistence. Rather, it is because they already possessed such differences that these close relatives have been able to maintain their separate identities. A precisely similar view has been expressed recently by Hubbs and Strawn (14): "Apparently mate selection is a major isolating factor in darters." It may be supposed that the original advantages conferred by distinctive breeding dress lay in the advertisement of the sex and state of maturity of individuals, thus facilitating the finding or selecting of mates or stimulating the mating act. This seems to be supported by the conspicuous sexual differences in the breeding dress. If the "purpose" of distinctive patterns is to distinguish between species or populations rather than between sexes, a single pattern for both males and females would be sufficient and would presumably be a simpler evolutionary attainment.

It is inferred, therefore, that the essential step in the initiation of the existing species of *Oncorhynchus* was the development of a distinctive appearance, at the time of breeding, by geographically isolated populations. This, indeed, appears to be still a main factor in maintaining isolation.

Although obviously unsatisfactory as general taxonomic criteria, the breeding colour patterns (and associated body proportions) come nearer to providing an absolute distinction between the species of *Oncorhynchus* than the morphological characteristics to which prominence is given in systematic keys. While morphological differences are sometimes regarded as more "fundamental" than colour differences, they may owe their origin to the prior appearance of the latter.

The time periods which we have allotted for the establishment of the genus *Oncorhynchus* and its various existing species appear to be rather shorter than those which contemporary opinion is inclined to regard as normal for such evolutionary steps, although reliable information in this field is still so scanty that firmly based conclusions are not possible. As envisaged here, *Oncorhynchus* became geographically isolated from *Salmo* between half a million and one million years ago. Some of the existing species would have a total evolutionary history of half this time or less. The actual periods of geographical isolation would have been much less. Certain considerations make it seem possible that *Oncorhynchus* could have evolved at a relatively fast rate. If, as we are led to think, the chief factor in maintaining reproductive isolation has been the possession of distinctive breeding dress, we can readily believe that recognizable changes in colour patterns need not have involved very complicated genetic changes and might therefore have been accomplished within the few thousand generations for which geographical separation probably persisted. Further, provided interbreeding can be avoided, the process of differentiation may be greatly speeded by contact between closely similar forms. Lack (19) has emphasized the importance of contact and the resulting competition in stimulating ecological divergence. The geographical intermingling which is shown by existing species of *Oncorhynchus*, and which we believe has occurred at intervals during their evolution, could have provided this stimulus in a high degree.

The existing situation (many species occupying a common geographical area and showing only a relatively slight degree of ecological divergence) is probably quite transitory in evolutionary terms. It could be expected that competition would either rapidly eliminate certain species or stimulate more pronounced ecological specialization—or both—and that relatively slight changes in climate might markedly affect the existing balance between species. It cannot be overlooked, however, that the Pleistocene epoch which produced the Pacific salmon also produced the unpacific predator whose ability to alter environments may play a part in determining the future course of events for *Oncorhynchus*.

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The Use of Antibiotics for the Control of Spoilage in the East Coast Fisheries

by

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• Part I Introduction and Use of Antibiotics on gutted cod and haddock aboard trawlers.

INTRODUCTION

In 1939 Dr. H. L. A. Tarr of the Technological Station of the Fisheries Research Board of Canada at Vancouver, first demonstrated the value of very small quantities of sodium nitrite as a preservative for fresh fish. After many years of experience with this preservative, both in the laboratories and under commercial conditions, the Department of National Health and Welfare agreed to amend their regulations, making sodium nitrite legal as a preservative for fresh fish in Canada. It has been estimated that by 1950 more than 90% of the fresh fillets shipped from the Atlantic Coast to Central Canada were being treated with sodium nitrite.

Approximately twenty years after this discovery of the usefulness of sodium nitrite, Dr. Tarr demonstrated the value of another group of materials, as food preservatives. He showed that certain antibiotics, formerly considered of value only for combatting infectious diseases, could also be used for the preservation of fish and other foods. This, as is now well known, has been heralded as another significant step in the control of spoilage in foods.

Since that time a great deal of research has been carried out in this field. Laboratories in almost every country of the world have been experimenting in the use of antibiotics as food preservatives, both in regard to their effectiveness and also attempting to evaluate the possibility of any detrimental results that may occur to those who consume the treated foods.

In September, 1956, the Department of National Health and Welfare legalized the use of the antibiotic chlortetracycline (usually called CTC for short) as a preservative for fish in Canada. Since then, similar permission has been given for the use of a second antibiotic, oxytetracycline (OTC). It is interesting to note that of the principal fishing countries of the world, Canada was not only the first, but up to the present time is the only country that has permitted the use of antibiotics on fish.

With the wealth of literature that is now available on this subject there is no need for a general discussion on the merits of antibiotics as preservatives or of the fears of those who oppose them. It is a fact, however, that antibiotics are not equally effective on all marine products and under all conditions of storage and processing. This article is written primarily for the East Coast fishing industry. It will be confined to the results that have been obtained in Halifax in tests with CTC, and to a much lesser extent with OTC, on cod, haddock and sole that have been caught and processed in the Atlantic Maritime provinces.

There are three principal things to be considered in discussing the use of these antibiotics on fish. The first is the toxicity or other harmful results that may affect those who consume the treated foods. This is principally the concern of the Public Health authorities. They now believe that sufficient evidence has been accumulated to show that there is no danger if the antibiotics are used within the limits prescribed by law. The second point is directly related to the first: how to be sure that the treated fish always contain less than the maximum amount permitted by law. The third and most interesting point to consider from the standpoint of the producer, is the value of these preservatives and the most effective way of using them.

Because of the type of fishing and processing carried out on this coast the subject of preservatives lends itself to a further subdivision; treatment of the round or gutted fish in the boats at sea and treatment of the fillets in preparation for market. These will be discussed in two separate sections.

Whole or gutted fish may be treated with antibiotics in several different ways; fish may be stowed in ice containing an antibiotic; they may be dipped in a solution, a solution may be sprayed on them; and they may be held in tanks of chilled sea water containing small amounts of antibiotics.

Our experience has been limited to antibiotic ice and antibiotic dips. Up to the present none of our fishing vessels have been fitted with tanks for sea water storage, although that is anticipated in the near future.

CTC ABSORBED FROM DIPS BY ROUND OR GUTTED FISH

As might be expected, the amount of CTC taken up by the fish muscle from a solution is less with the whole or gutted fish than it was with the fillet. Much of the antibiotic remains in the skin and epithelial membrances, limiting the rate of diffusion into the muscle beneath. This means that a considerable proportion of the antibiotic is removed when the fish is cut and skinned to produce the fillet.

In one set of tests medium sized, gutted haddock were dipped for one minute in solutions containing 4, 8, 20, 40 and 80 ppm. of CTC. Immediately after dipping, fillets cut from these fish showed no measurable amount of antibiotic. After six days in ice fillets were again cut from the gutted fish and tested for CTC. The results are shown in Table I. It may be seen that the muscle of the gutted fish contained very little antibiotic.

Increased keeping time of CTC dipped gutted fish

In the tests made with whole or gutted fish the keeping time has been defined as that period during which Grade I fillets (showing no off odours

TABLE I. CTC content of fillets that had been cut from medium sized gutted haddock. The gutted fish had been previously dipped for one minute in CTC solutions ranging from 4 to 80 ppm. and then stored six days in ice.

(Each figure is an average value from fifteen fillets).

CTC content of dipping solution (pm.)	CTC content of skinned fillets cut from gutted fish (ppm.)
4	0
8	0
20	trace
40	0.080
80	0.130

or other signs of deterioration) can be cut from the fish. It is realized that this is a very high standard but it leaves us with an easily recognized and clearly defined keeping time.

Table II gives the results from 12 different sets of tests where gutted cod and haddock were dipped into CTC solutions ranging from 4 to 80 ppm. In each case the immersion time was one minute and the fish were all stored in chipped ice. The results are somewhat disappointing when compared to the results of similar treatments given to the fillets. It suggests that for gutted fish, dips containing 5, 10 or 25 ppm. CTC are insufficient. Better results are obtained with 50 to 100 ppm., and even with 50 ppm. we cannot expect better than a three to five day increase in keeping time.

TABLE II. The keeping time of untreated, gutted cod and haddock stowed in ice and the keeping time of similar lots of fish dipped for one minute in solutions containing from 4 to 80 ppm. CTC before stowage.

Conc. of dipping solution (ppm.)	Species of fish	Keeping time of untreated control fish (days)	Additional keeping time due to CTC dip (days)
4	Medium haddock	9	0
8	Medium haddock	9	1
10	Market cod	6	1
10	Scrod cod	4	1
20	Medium haddock	9	3
25	Market cod	6	1
25	Scrod cod	4	2
25	Market cod	7	4
40	Medium haddock	9	4
50	Market cod	6	3
50	Scrod cod	4	2
80	Medium haddock	9	5

CTC absorbed from antibiotic ice by gutted fish

The usual concentration of either CTC or OTC in ice used for preserving fish is 5 ppm. For several days after gutted cod or haddock have been stowed in this ice very little antibiotic penetrates into the muscle. Following this there is a very slow buildup, but even after fourteen days the average content per fillet is less than 0.5 ppm. (see Table III). It may be seen that the antibiotic is mainly concentrated in the skin which is removed during the filleting.

Increasing keeping time of gutted fish in CTC ice

Sufficient CTC ice was put aboard a trawler to permit the icing down of 12,000 pounds of fish, using the proportion of one of ice to three of fish. A control pen, containing similar fish, caught at the same time, was iced down with ordinary ice. At the time of landing, part of the fish had been under ice for six days and the remainder for seven days. At this time some of the fish were examined and tested and some were transferred to bins in the Station's pilot plant, where they were carefully re-iced for further study.

At the time of discharge from the trawler, representative lots of fifteen fish each, were taken from corresponding locations in the pens containing the CTC and normal ices. Every fish was carefully and individually examined by five fresh fish inspectors and then tested for their trimethylamine content. The results are given in Table IV.

It is obvious that after six days in stowage most of the fish were in a good state of preservation. At seven days some of the fish were beginning to shift from first to second grade. Although the preference was slightly in favour of the fish in the CTC ice, the difference was not great. After nine days 85% of the fish in the ordinary ice were second grade and the remainder were Grade III. At this period 100% of the fish in the CTC ice were Grade II. After eleven days in ice all of the control fish were discarded as Grade III, while approximately 75% of those in the CTC ice were still Grade II.

These results indicate that for this particular trip, the stowage picture can be divided into two parts: (1) For a period of six days, there was little to choose from between the two lots of fish. During the next four days the control fish passed through Grade II and into Grade III. During this same period most of the fish in the CTC ice remained Grade II.

The increase in keeping time due to the CTC ice was not much more than one day if we limit the term to the persistence of high quality fish; on the other hand they remained "edible" by our standards for an additional four days. Other tests showed results in which the increase in keeping time ranged from one to three days, while the increase in the period when the fish was deteriorating, but still edible, went up to six days. The species of fish, the size of the fish, the season of the year and the care taken by the individual crews are all factors that contribute to those differences in keeping time.

It is of interest to note that other workers have obtained somewhat similar results with cod or haddock. Reports of tests made in Denmark with CTC ice shows there was a period of about ten days before the beneficial effect of the antibiotic ice became evident. The British workers at Torry give the period of ten to twelve days before there is any noticeable difference in the fish stowed in CTC ice and those stowed in ordinary ice. The Danes found that according to their standards the CTC ice added one or two days to the keeping time of the fish. The British believed that it added seven to ten days. One must realize however, that the British consider the limit of edibility of gutted cod and haddock in ordinary ice to extend up to fourteen to sixteen days. This is not said to cast any reflection on the British standards, but it must be understood when we attempt to interpret these comparative keeping times that have been reported by workers in other countries.

Antibiotics and bilgy fish

It was hoped by some that antibiotics might prove effective against the o'd bug-bear of bilgy fish. Theoretically it did not seem probable. CTC and OTC

TABLE III. The CTC content of fillets and skins cut from fillets. These were taken from gutted haddock that had been stowed in CTC ice (5 ppm.) for six to thirteen days before the fillets were cut out.

Portion tested	No. samples tested	Days in CTC ice	CTC content (ppm) Ave.	Min.	Max.
Skinned fillets	96	6	trace	(all under 0.01)	
Skinned fillets	14	9	trace	trace	0.16
Skinned fillets	14	11	0.14	0.01	0.52
Skinned fillets	14	13	0.21	0.01	0.56
Skins only	14	9	1.14	0.48	2.26
Skins only	7	13	0.72	0.37	1.42

TABLE IV. Showing the grades and mean trimethylamine values of fish that had been in the vessel six and seven days in normal ice and CTC ice and also for the same fish that were re-iced in boxes for two and four days after the vessel had been discharged.

STORAGE CONDITION.	Normal ice	CTC ice.
Fish from top levels of pens (6 days old)		
proportion of fish graded I	100%	100%
Fish from bottom level of pens (7 days old)		
proportion of fish graded I	66%	80%
proportion of fish graded II	33%	20%
mean TMA values	1.98	1.48
Re-iced fish (9 days old)		
proportion of fish graded II	85%	100%
proportion of fish graded III	15%	—
mean TMA values	3.8	3.0
Re-iced fish (11 days old)		
proportion of fish graded II	100%	75%
proportion of fish graded III	—	25%
mean TMA values	10.6	6.0

are bacteriostats not bacteriocides. This means that they delay the growth of bacteria but they do not kill them. As we have seen with the fillets, the heavier the initial contamination on the fish the less effective they are. Bilgy odours develop when fish are jammed tightly up against slimy water-soaked boards that are extremely heavily contaminated with spoilage bacteria. In this case the delaying action of the antibiotic is of little value.

In several experiments fish were purposely placed against wooden walls and shelf boards in pens that had CTC and normal ice. In both instances the fish became bilgy at approximately the same time. Fish dipped in solutions of 50 ppm. CTC and placed under the same conditions developed bilgy odours one half to one day later than the controls. It seems obvious that it is much safer to keep the fish away from the boards than to try to stop bilgy odours with antibiotics.

Conclusions regarding the use of antibiotic ice and antibiotic dips for gutted cod and haddock in the trawlers

From the standpoint of ease of application the antibiotic ice is more promising than dipping the fish on the vessels at sea. From the standpoint of increased keeping time, the dip is much more satisfactory.

From our tests we have concluded, that if very carefully used, ice containing 5 ppm. CTC or OTC might add one or two days to the keeping time of well iced cod and haddock. That it is not more effective appears to be due to the fact that the principal spoilage in the earlier stages of deterioration takes place in the gut cavity, where the water from the melted ice does not always penetrate. Somewhat similar results were obtained previously with nitrite ice, and quantitative tests for nitrite in the fish showed very uneven distribution with none at all in the gut cavity in the majority of cases. With both nitrite ice and CTC ice better results were obtained when the fish were carefully iced in boxes instead of the pens. In this case the fish are better iced, the ice is more evenly distributed and there is more chance of properly placing the fish on the ice.

As might be expected, antibiotic ice is of no value in preventing the development of bilgy odours in fish that are jammed up against heavily contaminated, soggy wooden surfaces.

The gutted fish that were dipped in antibiotic solutions showed more promising results. The concentrations necessary are at least 50 ppm., or more preferably 100 ppm., of either CTC or OTC. This is five to ten times stronger solution than is used for fillets and therefore that much more costly. The gutted fish, unless washed before dipping, are very much dirtier than the fillets, (because of the blood, slime and faeces) and the solutions would have to be changed more often. There would also be a much greater loss of solution on the deck because of the unsteadiness of the vessel and the physical difference between a gutted fish and a fillet.

However, most of the last five days catch now landed by our trawlers is in a good state of preservation. With more care and better icing this could be extended. Most of the spoilage takes place in fish that are in the hold for six or seven days or more. This suggests that antibiotic dips for gutted fish could be more profitably confined to the first caught fish, using solutions of 100 ppm. for fish that are likely to be in the hold for six to eight days.

It has been suggested by some that a dip, as well as antibiotic ice, should be used on all the fish in the vessels. This seems to us to be an idea from the minds of those trying to sell the antibiotic rather than from those who make a living by selling the fish.

There is much to be said for the storage of round or gutted fish in chilled sea water — if it can be adapted to our particular types of fishing and processing. If the CTC or OTC are added to the water in which the fish are more or less loosely suspended, there is every opportunity of these antibiotics coming continuously in contact with all the areas of the fish where spoilage is most active. Up to the present, however, we have had no experience with this type of storage.

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• Part II: The Effect of Antibiotics on the Keeping Time of Fillets.

THE literature dealing with the effect of antibiotics on the keeping time of fish and other marine products is sometimes very confusing. In the hands of different workers the same antibiotic apparently gives different results. One laboratory reports that dipping cod fish in a given concentration of antibiotic has added thirteen days to its keeping time, while another finds that using the same concentration of the same antibiotic increased the keeping time by only two days. There are some very good explanations for these apparent contradictions and it would be worth while to discuss the problem before giving our own results.

Basically the differences are a matter of definition. Some workers measure spoilage by the number of bacteria

that are present or that develop under given conditions; others measure spoilage by determining the amount of some specific chemical that usually accumulated in the muscle as spoilage progresses; still others rely more on organoleptic examination following the changes that take place in the fish by means of their senses of sight, smell and touch — and sometimes by their sense of taste. All these methods of following the development of spoilage are useful but the results do not always coincide. For example, in one carefully carried out experiment a Danish scientist treated fish with CTC to find out how much it would extend its keeping time. He came to these conclusions:

(a) Judged by chemical tests the CTC extended the keeping time by three to five days.

(b) Judged by bacterial counts it was extended by five to six days.

(c) Judged by examining the appearance, flavour and texture of the fish itself, the increase was only one or two days.

There is one other difficulty. This is the problem of deciding when fish muscle has reached the end of its keeping time. There is no need to point out that standards of quality for fresh fish vary; not only from country to country, but also from one part of a country to another part of a country. The same applies to scientific workers: there is no standard defining when a fish has reached the end of its keeping time.

If we cannot agree when a fish reaches the stage where it is no longer fit for commercial use, how then can we compare differences in keeping times?

The age and quality of the fillet at the time of treatment influences the extent to which its keeping time can be increased by a preservative. The fresher the fish, and the freer it is from contamination, the more effective will be the action of the antibiotic. Some of the apparent contradictions in the reports showing the length of time antibiotics have increased the keeping time are due to this difference in condition of the fish at the time of dipping. Two such reports read at one meeting is a typical example of this. One investigator started with fish not yet into rigor mortis. In fact, they were brought live into the laboratory and fillets cut out under conditions where special care was taken to keep down bacterial contamination. The other man worked with fish that had been in ice for six days before being filleted and they were cut and skinned with no particular care to prevent contamination. It should be no surprise that using the same antibiotic treatment the first lot of fish kept several days longer than the second lot.

TABLE V. Increase in the keeping time of twenty-one lots of CTC treated sole, cod and haddock fillets, compared with the keeping time of untreated fillets for each lot.

Species of fish	Conc. of CTC (ppm)	Dipping time (min)	Keeping time of untreated fillets (days)	Increased keeping time due to CTC (days)	Storage temp. (°F.)
sole	10	2	2	2	33° to 35
sole	25	2	2	4	33° to 35°
sole	50	2	2	5	33° to 35°
sole	28	1	3	13	32°
cod	10	1/2	3	4	32°
cod	10	1/2	3	3	32°
haddock	10	1/2	3	3	32°
cod	20	1/2	4	6	32° to 35
sole	10	1/2	5	4	32°
haddock	10	1/2	5	4	32°
cod	3	1	5	1	32° to 35°
cod	25	1	5	10	32° to 35°
cod	200	1	5	12	32° to 35°
cod	10	1/2	6	6	32°
haddock	10	1/2	6	8	32°
cod	10	1/2	7	8	32°
haddock	10	1/2	7	10	32°
haddock	10	1/2	7	8	32°
haddock	10	1/2	8	9	32°
haddock	10	1/2	9	8	32°
haddock	30	1	11	13	32°

THREE IMPORTANT POINTS

Therefore, to better understand and interpret the results of the tests made in this laboratory and reported here, three things must be clearly understood.

(1) It has been taken for granted that most of our fresh fish producers are more interested in how much longer a treatment will keep their fish in first grade quality, rather than how long the fish will remain in those stages where the spoilage odours are developing and the edibility of the fish is questionable. Instead of defining keeping time as the period during which a fish remains "edible" (which is only a matter of opinion), we have limited it to that period during which the fillet remains free from all spoilage odours, or during which the fillet remains Grade I quality. We have purposely excluded that period when the first spoilage odours are developing and when the fillet is edible but of inferior quality.

(2) Basically our judgment of the state of preservation of the fish was based on organoleptic examination by persons who have been trained in grading fish fillets. This was always supplemented by chemical tests and occasionally by bacterial counts. But whenever a fillet had developed off odours or showed other signs of deterioration it was down-graded regardless of the results of chemical or bacteriological tests.

(3) Our purpose has not been to see how long antibiotics will preserve fish under ideal conditions that can only be attained in the laboratory, but rather to see how long they will prolong the keeping time of fillets produced under present day commercial conditions in our local plants. These plants process fish that have been iced in boats from one to seven or eight days before they are filleted. We were aware that we were not dealing with fish of uniform quality.

Table V shows the results from tests where twenty-one lots of commercially prepared fillets were dipped in various concentrations of CTC solution and stored either at 32°F. or in a cold room where the temperature varied from 33°F. to 35°F. It is of interest first to note that great difference in the keeping time of the control, untreated fish. This ranged from three to eleven days at 32°F. It may be seen that where 10 ppm. of CTC was used in the dipping solution the keeping time was just about double that of the untreated fillets. It may also be seen that this initial quality of the fish at dipping time is of more importance than either the concentration of the dip or the period during which the fish are immersed.

The fish used in Table V were purposely selected to show what occurs when fish of varying degrees of quality were used. Experience has indicated that most of the cod and haddock fillets from the better plants in the Maritimes have a keeping time of six to nine days at 32°F. (i.e. it remains Grade I quality. They may be saleable, but with decreased quality, for several additional days). Fillets of sole have a shorter keeping time, while for halibut it is often very much longer.

In relation to the dipping of fillets

in antibiotics the question arises as to how long a solution can be used and remain effective. Our tests with sole and cod fillets indicate that the strength of CTC in a solution is depleted very slowly. The same goes for OTC. We made three different measurements to determine the effect of continuous dipping on the strength of a CTC solution.

(1) Periodically fillets were taken after being dipped and their CTC content determined to see if it decreased with time.

(2) We compared the keeping time of the first dipped fillets with those that were dipped later to see if the latter showed any decrease.

(3) We made periodic tests of the CTC content of the dipping solution as it was being used. The results showed that the first dipped fish took up approximately the same amount of CTC as those after fifteen, thirty, forty-five and sixty minutes continuous dipping. Also, the fillets dipped after forty-five and sixty-five minutes had the same keeping time as those dipped at the beginning. The slight decrease in the concentration of the solution itself was no more than could be accounted for by dilution through the continuous introduction of moist fish. These results simply indicate that unlike nitrite, the length of time that a CTC or an OTC solution is used is not critical. It is more likely that the solution will be discarded because of the accumulation of debris from the fillets than because of depletion of the strength of the solution. One can with impunity follow the recommendations of the manufacturers of CTC and OTC regarding the amount of fish that should be dipped in a given volume of solution.

CTC vs OTC for fillet dips

Because of the potential financial implications to the company who can monopolize the market, there is a very vigorous competition between the manufacturers of antibiotics as food preservatives. Many claims and counter claims have been made for CTC and OTC as fish preservatives. Dr. Tarr and his co-workers in Vancouver consistently get better results from CTC. On the other hand Stern and his co-workers at the College of Fisheries at the University of Washington in Seattle found no significant difference between the preservative value of CTC and OTC. Most of our work at Halifax has been done with CTC because it was used by the industry on the East coast and for a time it was the only antibiotic permitted by law for use on fresh fish. Several direct comparisons using equal concentrations of CTC and OTC in dips for cod fillets have been made. The preference, although very slight, has been in favour of the CTC. For example: — untreated cod fillets remained Grade I for four days; treated with CTC they remained in the same state of preservation for nine to ten days which was comparable with eight to nine days for the OTC. The point that seems most important to us, is not that one product was so much better than the other, but that either one, properly used, made a very decided improvement in the keeping time of the fillets.

Nitrite vs Antibiotics as Preservatives

We have been frequently asked to make a comparison between nitrite and CTC or OTC as preservatives for fresh fish. To this we cannot give an answer unless the questioner states what strength of nitrite solution is to be compared to what strength of antibiotic solution.

In the case of nitrite, we ordinarily use a concentration in the dip that will leave the fillets with something close to the maximum 200 ppm. permitted by law. With the antibiotic the concentration that is usually recommended is considerably below that which would leave the fillets with the maximum amount permitted by law. In commercial practice with fillets of cod and haddock a solution containing 10 ppm. of either CTC or OTC is usually recommended.

Several tests, using these customary amounts of nitrite (0.2% solution) and CTC (10 ppm.) have been made and the keeping time of the fillets was determined. In every case the nitrite treated fillets kept longer than the contro!s, and in every case the CTC treated fillets kept longer than the nitrite treated fillets. In some instances the CTC treated fish kept only one or two days longer than the nitrite treated fillets at 32°F., while in others the difference amounted to seven or eight days.

There seems no doubt that if increased keeping time is the only consideration, either CTC or OTC does a better job than sodium nitrite.

Conclusions concerning the value and limitations of CTC and OTC for fillets.

The results of these numerous tests leave no doubt that, properly used, these two antibiotics are the most efficient of the preservatives now available for increasing the keeping time of fillets. The concentration in the dips that is now being recommended (10 ppm.) is such that there is very little likelihood of the fillets taking up more than the maximum amount permitted by law. In fact this concentration could doubled or trebled, if necessary, without running into danger on this point.

We recommend the use of either CTC or OTC as preservatives for fresh fillets. At the same time we also feel that all plant operators should be aware of the limitations of these products.

It cannot be over emphasized that the value of CTC or OTC in extending the keeping time of stored fillets is dependent upon the quality of the fish at the time it is dipped. Whether it adds two days or sixteen days to the life of the fillet will be determined by the initial condition of the fillet and the extent of its bacterial contamination.

It should also be realized that antibiotics cannot add one iota to the quality of the fish you start with. Poor quality fish dipped in an antibiotic solution will remain poor quality fish. The only advantage of the dip is to retard further deterioration.

Experience has shown that it is possible to process, pack and ship fillets to Montreal from Halifax or Lunenburg, without an antibiotic, and have them reach the consumer in first class condition. This is especially so in the late winter and early spring. In fact there may be no observable difference in treated and untreated fish for several days or even a week after the time of shipment. It is not true that "you have never tasted real fresh fish until you have tasted fish treated with an antibiotic". Such statements are true only if you live in a location so remote from the source of supply that it is impossible to bring fish in without having it deteriorate during transit. It is quite possible, for instance, to ship Atlantic cod and haddock to Montreal and have it on the market or in the retail stores in Grade I condition, without the use of an antibiotic. The advantage of the antibiotic is that this good quality will last longer.

Because of the wide and enthusiastic publicity given to antibiotics shortly after they became available in Canada, some processors failed to realize that even these better preservatives have their limitations. In many instances CTC treated fish were held beyond their normal keeping time and customers got well advertised fish that was spoiled or spoiling. This of course was no fault of the preservative, but it is an indication of the exaggerated idea of the value of antibiotics that some persons acquired from reading "popular" articles on the subject during that particular period.

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• Part III: Factors Affecting the Amount of Antibiotic Taken up by Fillets

THERE are several things that influence the amount of antibiotic taken up by fish muscle from a dipping solution. The first is the concentration of the antibiotic in the solution. But as well as that, the length of time the fish are immersed, the temperature of the dip, the size of the fillet and the physical condition of the fillet (i.e. whether it is soft or flaked apart) must also be considered.

To begin with, as a very general statement, our experience has shown that under the usual plant conditions, fillets pick up amounts of antibiotic in the range of 1/10 to 1/50 of that of the dipping solution. For example, if fillets are being dipped in a solution containing 20 ppm. CTC we might expect the average fillet to contain somewhere between 0.5 and 2.0 ppm. CTC.

Each fillet does not pick up exactly the same amount of antibiotic from a solution. For example, in one test, cod fillets of approximately the same size were dipped in CTC solution for exactly the same period of time. Afterwards twelve fillets were measured for their CTC content. The average value was 1.51 ppm., but the values for the individual fillets were: 0.68, 0.72, 0.76, 1.36, 1.48, 1.56, 1.60, 1.76, 1.96, 2.00, 2.00 and 2.16 ppm. This shows the inadvisability of trying to find out the CTC content of dipped fillets by measurements on a single fillet. It also shows that where fairly high concentrations of the CTC are being used, we may run into the problem of having certain individual fillets with CTC concentrations above that permitted by law, while the average value is satisfactory.

Let us see what happens if we repeat this test many times over with different lots of fish taken directly from the end of a cutting line. Table VI shows the average minimum and maximum amounts of CTC picked up by 14 different lots of fillets over a period of several months. It may be seen that the dip concentrations varied from 10 to 50 ppm. and that the immersion time varied from one-half to two minutes. Longer immersion periods were omitted because they were not feasible under commercial operation conditions. The figures indicate that if fillets are given short dips (up to two minutes) in solutions containing not more than 30 ppm. the maximum amount of CTC picked up by individual fillets will be well within the 5 ppm. permitted by law.

TABLE VI. Fourteen lots of sole and cod fillets were dipped into CTC or OTC solutions having concentrations of 10 to 50 ppm. This table shows the amounts of antibiotics picked up by the fillets and given as the average value for each lot, and also the values for the fillets with the least and the most antibiotics for each lot.

Conc. of Antibiotic (ppm)	Dipping time (min)	Fish Species	Concentration of antibiotic in fillets in ppm. Average	Minimum	Maximum.
50	1	sole	2.44	0.88	3.76
50	2	sole	2.17	0.72	4.08
50	2	sole	2.03	0.40	2.76
30	1	cod	2.40	1.54	3.18
30	1	cod	2.33	1.40	3.24
28	1	sole	2.78	1.76	4.80
25	2	sole	1.42	0.68	2.60
25	1	cod	1.32	0.41	2.53
25	2	sole	1.51	0.68	2.16
20	½	cod	1.01	0.88	1.12
20	1	cod	0.98	0.63	1.41
10	½	cod	0.68	0.37	1.01
10	2	sole	0.53	0.21	1.08
10	2	sole	0.43	0.21	1.00
10	1	cod	0.27	0.01	0.87

IMMERSION PERIOD

Within considerable limits, the length of time the fillets are immersed makes very little difference in the amount of antibiotic taken up by the fillets. Under commercial conditions where all sorts of other variations exist, we have not been able to find any significant difference in dips of one-half to two minutes. Under rigidly controlled conditions in the laboratory, extending the immersion period from one-half to five minutes approximately doubled the average value for large cod fillets and increased the content in very small sole fillets by more than three times. In experiments reported by others it has been shown that immersion periods up to one hour did little more than double the antibiotic content.

A somewhat similar relationship exists between the size of the fillets and the concentration of CTC picked up from a solution. In theory the larger and thicker the fillets the less antibiotic will be picked up. In the laboratory small fillets, (average four ounces) of sole and large fillets of cod (average three pounds) were immersed for one-half minute in a CTC solution containing approximately 10 ppm. after dipping the average CTC value or the sole fillets was 1.2 ppm.; that of the large cod fillets was 0.8 ppm. However, the physical condition of the fillets complicates this relationship. Fresh, firm, even fillets take up less antibiotics than those which are soft, ragged and flaked open. In normal plant operation it is more difficult to demonstrate this relationship between size and CTC pick up unless special care is taken to select uniform fish.

TEMPERATURE EFFECT

Our previous experience with nitrite dips has shown that the temperature of the dipping solution made a considerable difference in the amount of nitrite picked up by the fillets. As the solution warmed up more nitrite went into the fish muscle. Something the same occurs with antibiotics, although to a lesser extent. This can best be understood by an example. A solution containing 30 ppm. CTC was divided into three parts. The first was chilled to 34°F. the second to 45°F. and the third to 77°F. Medium sized cod fillets were immersed for exactly one minute in each of the solutions. The fillets themselves were approximately 46° to 48°F. at the time of dipping. The proportion of fish to solution was 90 pounds of fillets in 15 gallons of solution. After being left to drain for ten minutes the CTC content of nine fillets taken from each lot was measured. The results are given

in Table VII. There was little difference in the CTC content of the fish dipped in the solutions at 45° and 77°F., but there was considerably less in the fillets from the solution that was close to freezing temperature.

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TABLE VII. The CTC values of 29 individual medium sized cod fillets, one third of which had been dipped in a CTC solution at 34°F., one third in a solution at 45°F., and one third at 77°F.

Fillet	CTC content in ppm of fillets dipped in CTC solutions at:		
	34°F	45°F.	77°F.
1	1.96	2.96	1.44
2	1.12	2.80	1.68
3	2.44	2.36	2.64
4	1.40	2.92	2.04
5	1.96	3.20	1.68
6	1.76	2.00	3.20
7	1.56	2.24	2.24
8	1.68	1.56	3.24
9	1.56	2.48	2.24
Average	1.71	2.50	2.33

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